RESPONSE TO COMMENTS

EPA-HQ-OPP-2019-0274

I. Comments on Potential for Introgression of OX5034 Genes into the Local Wild Aedes aegypti Mosquito Population

Several commenters stated that OX5034 genes will enter the local wild Aedes aegypti mosquito population in the test area and form a triple hybrid mosquito population given that OX5034 itself is a hybrid strain containing the genetics of mosquitoes isolated from Cuba and a Latin strain from the Instituto Nacional de Salud Publica (INSP), Mexico.

These commenters raised concerns about possible risks associated with hybridization of OX5034 with the native Aedes aegypti mosquito population in the test area. The concerns revolve around possible effects associated with: (1) genes encoding the genetic characteristics of the mosquito strain (Rockefeller/Latin hybrid) engineered to create OX5034 spreading in the native Aedes aegypti mosquito population, and (2) the genetic material expressing the active ingredient and inert ingredients, tTAV-OX5034 and DsRed2-OX5034 respectively, inserted into that mosquito strain to create OX5034 spreading into the native Aedes aegypti mosquito population.[1] Commenters pointed to the recent study by Evans et al[2]. and stated that there is no guarantee that only beneficial traits would introgress into the native local Aedes aegypti mosquito population. Commenters indicated that according to the Evan et al study, the amount of OX5034 genetic material introgressed into the native Aedes aegypti population was not trivial ranging from 10 to 60%.

A. Will Genes from OX5034 Enter the Gene Pool of the Local Wild Aedes aegypti Mosquito Population

Three commenters (Center for Food Safety 0344, GeneWatch UK 0335, C. Cheromiah 0266) pointed out the likely genetic background of OX5034. For example, the Center for Food Safety stated that:

---

[1] The active ingredient of the OX5034 *Aedes aegypti* mosquito ("OX5034" or "OX5034 *Aedes aegypti")* is a tetracycline-repressible transactivator protein variant (tTAV -OX5034) and the genetic material necessary to produce the protein *in vivo* in female offspring of OX5034 *Aedes aegypti* matings. Female progeny inheriting the OX5034 rDNA construct express the tTAVOX5034 protein as larvae and, in the absence of tetracycline or its analogues, die in L2/L3 larval instal' stages, while males survive to fully functional adulthood. This means that released OX5034 *Aedes aegypti,* reared in the absence of tetracycline, will be males that cannot bite humans or other animals, and do not transmit disease. The inert ingredient in OX5034 *Aedes aegypti* is a fluorescent marker, DsRed2-0X5034, which aids in the detection of *Aedes aegypti* carrying the #OX5034 rDNA construct. The DsRed2 protein belongs to a family of red fluorescent proteins, which are members of a group of fluorescent proteins identified in several *Anthozoa* species. DsRed2 is a synthetically modified variant of the original red fluorescent protein isolated from a coral-like anemone, *Discosoma* spp.

[2] Evans, B. R., Kotsakiozi, P., Costa-da-Silva, A. L., Ioshino, R. S., Garziera, L., Pedrosa, M. C., Malavasi, A, Virginio, J. F., Capurro, M and Powell, J. R. (2019). Transgenic *Aedes aegypti* Mosquitoes Transfer Genes into a Natural Population. *Scientific Reports*, **9**(1), 1–6. https://doi.org/10.1038/s41598-019-49660-6

> "Oxitec's GE mosquitoes have been developed from a non-native strain (the Rockefeller laboratory strain[3], originally from Cuba[4]). In the Cayman Islands, this was backcrossed into a Mexico-derived genetic background[5] and it appears that this same strain was then used in Brazil and probably also in Panama. As described in Oxitec's draft Environmental Assessment for OX513A, originally submitted to the FDA, (pages 21 and 22), the GE strain OX513A was produced in 2002 by microinjection into individual embryos of *Aedes aegypti* from a Rockefeller strain background[6]. The strain was made homozygous by repeated back-crossing and then the insert was introgressed into an *Ae.aegypti* Latin strain background from Instituto Nacional de Salud Publica (INSP), Mexico. The Rockefeller strain is a common laboratory strain of *Aedes aegypti*, which appears to have been derived from a strain established in Havana, Cuba, by Carlos J. Finlay in 1881, used in the original experiments which established that *Aedes aegypti* mosquitoes are a vector for Yellow Fever[7],[8]. " (Center for Food Safety 0344 p. 11)

Three commenters (GeneWatch UK 0335, Center for Food Safety 0344, Anonymous 0224,) noted that the Oxitec's stated intent was that Rockefeller/Latin genes from OX5034 mosquito would enter the local wild mosquito population.

Anonymous (0224) explained that:

> "The idea is that mass releases of GM males will mate with wild females and their offspring will contain the femalekilling trait. This genetically engineered trait is intended to make most of the female offspring of these matings die before adulthood; however the male offspring are intended to survive and breed for multiple generations. In addition, wild female pests that have mated with the released GM males will lay eggs that inherit the GM female-killing trait . . . ." (Anonymous 0224, p. 13) [Typographical error in the original]

GeneWatch UK (0335) noted that:

> ". . . , because the OX5034 strain is female-killing only, GE males are expected to survive for multiple generations and this will considerably increase the spread of genes from the introduced strain into the wild population. In an online presentation, Oxitec presents

---

[3] Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ et al. (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology*, **5**: 11. doi:10.1186/1741-7007-5-1. http://www.biomedcentral.com/1741-7007/5/11

[4] Kuno, G. (2010). Early History of Laboratory Breeding of Aedes aegypti (Diptera: Culicidae) Focusing on the Origins and Use of Selected Strains. *Journal of Medical Entomology*, **47**(6), 957–971.

[5] Harris AF et al. (2011) Field performance of engineered male mosquitoes. *Nat. Biotech.*, 29(11), 1034-1037.

[6] Oxitec (2016) Draft Environmental Assessment for Investigational Use of *Aedes aegypti* OX513A. https://www.fda.gov/downloads/animalveterinary/developmentapprovalprocess/geneticengineering/geneticallyengineeredanimals/ucm487377.pdf

[7] Kuno G (2010) Early History of Laboratory Breeding of *Aedes aegypti* (Diptera: Culicidae) Focusing on the Origins and Use of Selected Strains. *Journal of Medical Entomology*, **47**(6), 957-971.

[8] Yellow Fever and the Reed Commission 1898-1901. University of Virginia Claude Moore Health Sciences Library. Historical Collection. http://www.hsl.virginia.edu/historical/medical_history/yellow_fever/index.cfm

> this as a benefit because it argues that the released laboratory-derived strain will spread insecticide susceptibility genes into the wild mosquito population[9]: however, there is no guarantee that only beneficial and no harmful traits will be spread in this way." (GeneWatch UK 0335 p. 1)

Center for Food Safety (0344) stated that:

> "When Oxitec's GE mosquitoes breed with wild mosquitoes some of their other genetic characteristics will be passed on to the local wild mosquito population." (Center for Food Safety 0344 p. 11)

GeneWatch UK (0335) pointed out that:

> " . . , Oxitec has demonstrated the effects of rapid introgression of insecticide-susceptible traits in its own research and modelling of its GE agricultural pests[10],[11]." (GeneWatch UK 0335 p. 9)

Other commenters (GeneWatch UK 0335, Center for Food Safety 0344, Florida Keys Environmental Coalition 0331, L.M. Castro 0332, GMO Free USA 0326) posited that data developed on the GE mosquito product, OX513A, that had been released into the environment during field testing, support the conclusion that OX5034 genes would introgress into the local wild Aedes aegypti mosquito population to form a hybrid Aedes aegypti mosquito population. For example, one commenter (GeneWatch UK 0335 p. 1) noted that with OX513A:

> "The released GE males mate and produce offspring which inherit the genetically engineered late-lethality trait. This means that most (but not all) of the GE mosquitoes' offspring die at the late larval stage, in the water where the female mosquitoes lay their eggs. GeneWatch UK has repeatedly warned . . . that this partial survival rate, even if low (a reported 3 to 4% in laboratory conditions), would lead to the establishment of hybrid mosquitoes in the environment, which might possess altered properties, including the potential for enhanced disease transmission or resistance to insecticides. A recent paper, reporting monitoring of wild mosquito populations following some of Oxitec's experiments in Brazil, has confirmed that such hybrid mosquitoes have indeed

---

[9] Martin-Rendon, E (2019) Introduction to Oxitec 2nd Generation Mosquitoes Technology Summary - Roll Back Malaria Vector Control Working Group – Geneva, 30th Jan - 1st Feb, 2019. https://endmalaria.org/sites/default/files/Enca%20Martin-Rendon.pdf

[10] Harvey-Samuel, T., Morrison, N. I., Walker, A. S., Marubbi, T., Yao, J., Collins, H. L., ... Alphey, L. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biology*, **13**, 49. http://www.biomedcentral.com/1741-7007/13/49

[11] Alphey N, Bonsall MB, Alphey L (2009). Combining pest control and resistance management: synergy of engineered insects with Bt crops. *Journal of Economic Entomology*, **102**(2), 717–732.

> spread into the area surrounding the release sites.[NOTEREF _Ref30598723 \h * MERGEFORMAT]" (GeneWatch UK 0335 p. 1)

The Florida Keys Environmental Coalition (0331) also noted the recent Evans et al study[NOTEREF _Ref30598723 \h * MERGEFORMAT] and commented that:

> "Oxitec could not see where the[y] lab results showing that 15% of the OX513A survived 42 days, "long enough for females to take 2 blood meals and lay 2 clutches of eggs" would suggest survivability, likely without the fluorescent marker genetically inserted and hybridization with wild indigenous mosquitoes was expectable. Yet, this was shown to be true in the attached nature.com, Yale study, recently reported from the Brazil trial of the OX513A in Jacobina (attached as PDF)."[NOTEREF _Ref30598723 \h * MERGEFORMAT] (Florida Keys Environmental Coalition 0331 p. 2) [Typographical error in original]

The Center for Food Safety (0344) stated that:

> "A recent paper, reporting monitoring of wild mosquito populations following some of Oxitec's experiments in Brazil, has confirmed that such hybrid mosquitoes have indeed spread into the area surrounding the release sites[NOTEREF _Ref30598723 \h * MERGEFORMAT]. Because the OX5034 strain is female-killing only, GE males are expected to survive for multiple generations and this will considerably increase the spread of genes from the introduced strain into the wild population." (Center for Food Safety 0344 p. 11)

GeneWatch UK (0335) echoed this comment stating that:

> ". . . due to the survival of GE males for multiple generations, the OX5034 strain is expected to increase, rather than reduce, the spread of genes from the released GE non-native strain into the wild *Aedes aegypti* mosquito population, compared to the OX513A strain." (GeneWatch UK 0335 p. 2)

L.M. Castro (0332) stated that:

> "A risk-benefit analysis of the proposed release of genetically modified mosquitos suggests that a significant risk of genetic material transfer exists in connection to this Experimental Permit to Combat Mosquitoes. This assertion is supported by the work of researchers from Yale University and Brazil (Evans et al.[NOTEREF _Ref30598723 \h * MERGEFORMAT]). They report that "genetic sampling from the target population six, 12, and 27–30 months after releases commenced provides clear evidence that portions of the transgenic strain genome have been incorporated into the target population" and "release of the OX513A has led to significant transfer of its genome (introgression) into the natural Jacobina population of *Aedes aegypti*. The degree of introgression is not trivial. Depending on sample and criterion used to define unambiguous introgression, from about 10% to 60% of all individuals have some OX513A genome (Tables 1 and E1).

> Even if the strain of Oxitec mosquitoes released in the USA is not the same as the one released in Brazil, the point is that the failings of the proposed experiment will only be known after-the-fact and the impacts will most likely be irreversible." (L.M. Castro 0332 p. 1) [Footnote referencing Evan's *et al* paper inserted]

Anonymous (0329) argued that Oxitec's claims that "its GM mosquitoes are self-limiting and will result in a sustainable decrease in the wild population" are not reliable:

> "According to a peer reviewed Yale study[NOTEREF _Ref30598723 \h * MERGEFORMAT], the GM mosquitoes released in Brazil reproduced and their GM genes contaminated the wild mosquito population. The Yale study also found that the release in Brazil did not result in a sustainable decrease in the mosquito population." (Anonymous 0329 p.1) [Footnote referencing Evan's et al paper inserted]

GMO Free USA (0326), requesting an extension of the comment period, echoed the importance of the recent paper by Evans et al[NOTEREF _Ref30598723 \h * MERGEFORMAT] stating that:

> "New research has been published this week on the efficacy of a release of Oxitec's genetically engineered mosquitoes on mosquito populations in Brazil. The study, published by Yale University scientists in the journal Nature, documented unexpected and unintended consequences from the release. Not only did mosquito population numbers bounce back up in the months following the test, but some of the native mosquitoes retained genes from the engineered mosquitoes. [HYPERLINK "https://www.nature.com/articles/s41598-019-49660-6"] " (GMO Free USA 0326 p.1)

Several commenters (Anonymous 0226, Friends of the Earth 0342, GeneWatch UK 0335) raised other potential concerns.

Anonymous 0226 noted that:

> ". . . , concerns about the spread of the GM trait and other traits of the introduced strain will increase if GM males survive and breed for multiple generations. Depending on the details of the technology used, other new concerns may be identified. In caged experiments in Mexico using an earlier female-killing version (Oxitec's flightless female GM mosquitoes), the GM mosquito line was reportedly contaminated, so that half the GM females could fly and mate, rather than being unable to survive and reproduce[12]." (Anonymous 0226 p.13)

[12] Humans are genetically modifying mosquitoes to fight a disease we helped create. Quartz. 6 May 2015. http://qz.com/384874/humans-are-geneticallymodifying-mosquitoes-to-fight-a-disease-we-helped-create/

B. Comments on Whether Potential Problems Might Arise from Introgression of OX5034 Genetic Material into the Local Wild Aedes aegypti Mosquito Population

Several commenters argued that introgression of Rockefeller/Latin OX5034 genetic material into the local wild Aedes aegypti mosquito population could present problems that should be taken into consideration. Concerns identified with the introgression of OX5034 genes into the local wild Aedes aegypti population include: the hybrid population might have greater vector competency than the original local wild Aedes aegypti population, and the hybrid population might display hybrid vigor.

The Center for Food Safety (0344) stated that while Oxitec presents the spread of OX5034 genes into the local wild Aedes aegypti mosquito population as a benefit because ". . . the released laboratory-derived strain will spread insecticide susceptibility genes into the wild mosquito population[NOTEREF _Ref26803807 \h * MERGEFORMAT]":

> ". . , there is no guarantee that only beneficial and no harmful traits will be spread." (Center for Food Safety 0344 p. 11)

1. Comments Positing Potential Consequences of Genes of the Rockefeller/Latin Mosquito Strain Used to Create OX5034 Introgressing into the Local Wild Aedes aegypti Population

Several commenters (Center for Food Safety 0344, GeneWatch UK 0335, Anonymous 0226, Anonymous 0199) suggested EPA should evaluate the ramifications potentially associated with introgression of genes of the mosquito strain used to create OX5034 into the local wild Aedes aegypti mosquito population.

Anonymous (0226) noted that:

> "To create its GM mosquitoes, Oxitec started with a strain of Aedes aegypti mosquito that is commonly kept in laboratories, which probably originally came from Cuba. Before releasing the GM mosquitoes into the environment, it crossed them with wild strains from Mexico (for releases in the Americas) or Asia (for releases in Malaysia). When Oxitec's GM mosquitoes breed with wild mosquitoes some of their other genetic characteristics will be passed on to the local wild mosquito population." (Anonymous 0226 p. 11)

i. Comments raising concerns about hybrid vigor and disease transmission

The Center for Food Safety (0344) argued that:

> "The use of a non-native strain risks spreading altered disease transmission properties into the wild mosquito population and/or creating strains which exhibit "hybrid vigour"

> (for example, becoming more fertile, as has been demonstrated for hybrid strains of other mosquito species[13])." (Center for Food Safety 0344 p. 11)

Quoting José Maria Gusman Ferraz, a researcher at Ecological Engineering Laboratory of Unicamp (the University of Campinas, a public research university in the state of São Paulo, Brazil), L.M. Castro (0332) added that:

> "The study shows that there was a gene exchange, and that in this exchange the wild mosquitoes incorporated genes from another [transgenic] variety, resulting in hybrid insects, which usually have greater vigour and are more potent . . . . What we have now is a 'super mosquito' that can grow in environments where others might not grow[14]." (L.M. Castro 0332 p. 1-2) [L.M. Castro (0332) noted that this was translated from the original Portuguese by GMWATCH[NOTEREF _Ref27149257 \h * MERGEFORMAT],[15]]

The Center for Food Safety (0344) and GeneWatch UK (0335) went on to argue that:

> "Different strains of the same species are found in different places and some strains are more resistant to insecticides than others or better transmitters of disease (the four serotypes of the dengue virus and/or other viruses, such as chikungunya, zika and Yellow Fever). *Aedes aegypti* may transmit zika, chikungunya, yellow fever and four different serotypes of dengue, yet strains may vary significantly in their ability to

---

[13]Ekechukwu, N. E., Baeshen, R., Traorè, S. F., Coulibaly, M., Diabate, A., Catteruccia, F., & Tripet, F. (2015). Heterosis Increases Fertility, Fecundity, and Survival of Laboratory-Produced F1 Hybrid Males of the Malaria Mosquito Anopheles coluzzii. *G3* (Bethesda, Md.), **5**(12), 2693–2709. https://doi.org/10.1534/g3.115.021436

[14] https://gmwatch.org/en/news/latest-news/19144-guinea-pig-population

[15] "Troca de genes. "O estudo mostra que houve uma troca de genes, e que nessa troca os mosquitos comuns incorporaram genes de uma outra variedade, transgênica, resultando em insetos híbridos, que geralmente têm maior vigor, são mais potentes, sobre os quais ainda não há estudos. Muito menos quanto à sua eficiência na transmissão de vírus, que pode inclusive ser maior. O que temos agora é um 'super mosquito', mais resistente, que pode se desenvolver em ambientes em que outros talvez não se desenvolveriam", avalia o biólogo José Maria Gusman Ferraz, pesquisador do Laboratório de Engenharia Ecológica da Unicamp e professor da pós-graduação do Centro Universitário da Fundação Hermínio Ometto (UniAraras)."
https://www.redebrasilatual.com.br/destaques/2019/09/irresponsabilidade-da-ctnbio-produz-super-mosquito-da-dengue/

> transmit these tropical diseases[16],[17],[18],[19],[20],[21],[22],[23]. In the case of zika, little is known about vector strain variation and its consequences. The possible introduction of such traits needs to be considered very seriously. Harm to people's health can be increased if some serotypes or viruses can be transmitted more easily by the introduced strain than they were by the wild species already in the area, or if the strain is resistant to insecticides." (Center for Food Safety 0344 p. 11)

Anonymous (0199) stated that:

> "The demonstration of introgression resulting in increased genetic variation in Jacobina aegypti is a monumental setback. Oxitecs response is classic typology that introduced Gene's have no consequences and they assert that there is no danger because it is one species. A huge literature of nearly 50 years has validated significant population genetic differentiation between aegypti populations around the world and documented population variation in vector capacity for dengue, Zika and yellow fever viruses, mosquito behavior and insecticide resistance. To assert species specific variation is all the same is naive at best." (Anonymous 0199 p. 1)

J. Butler (0135) stated that:

> "Recently, the public has learned that Oxitec's GM mosquitoes released in Brazil bred and reproduced 'super mosquitoes 'and have become an alarming environmental hazard. These GM mosquitoes successfully reproduced and the resulting hybrid population is now spreading out of control, are more difficult to control, and, therefore, more prone to carry mosquito born[e] diseases." (J. Butler 0135 p. 1) [Typographical error in original]

---

[16] Bonizzoni M, Dunn WA, Campbell L,Olson KE, Marinotti O, James AA (2012) Strain Variation in the Transcriptome of the Dengue Fever Vector, *Aedes aegypti*. *G3*, **2**(1),103-114. http://www.g3journal.org/content/2/1/103.full

[17] Van Den Hurk AF et al. (2011) Vector Competence of Australian Mosquitoes for Yellow Fever Virus. *The American Journal of Tropical Medicine and Hygiene*, **85**(3), 446–451.

[18] Aitken TH, Downs WG, Shope RE (1977) *Aedes aegypti* strain fitness for yellow fever virus transmission. *The American Journal of Tropical Medicine and Hygiene*, **26**(5 Pt 1), 985–989.

[19] Tabachnick WJ et al. (1985) Oral Infection of *Aedes Aegypti* with Yellow Fever Virus: Geographic Variation and Genetic Considerations. *The American Journal of Tropical Medicine and Hygiene*, **34**(6), 1219–1224.

[20] Lima RS Jr, Scarpassa VM (2009) Evidence of two lineages of the dengue vector *Aedes aegypti* in the Brazilian Amazon, based on mitochondrial DNA ND4 gene sequences. *Genetics and Molecular Biology*, **32**(2), 414–422.

[21] Scarpassa VM, Cardoza TB, Cardoso RP (2008) Population Genetics and Phylogeography of *Aedes Aegypti* (Diptera: Culicidae) from Brazil. *The American Journal of Tropical Medicine and Hygiene*, **78**(6), 895–903.

[22] Vega-Rúa A, Zouache K, Girod R, Failloux A-B, Lourenço-de-Oliveira R (2014) High Level of Vector Competence of *Aedes aegypti* and *Aedes albopictus* from Ten American Countries as a Crucial Factor in the Spread of Chikungunya Virus. *Journal of Virology*, **88**(11), 6294–6306.

[23] Gonçalves CM, Melo FF, Bezerra JM, Chaves BA, Silva BM, Silva LD, ... Pimenta PF (2014) Distinct variation in vector competence among nine field populations of Aedes aegypti from a Brazilian dengue-endemic risk city. *Parasites & Vectors*, **7**, 320. http://doi.org/10.1186/1756-3305-7-320

J. Smith (0095) stated that:

> "Oxitec mosquito, OX513A, shows that Oxitecs claims that its GM mosquitoes are self-limiting are unreliable. The GM mosquitoes ended up breeding with native mosquitoes, transferring their genes into the natural population and forming hybrid mosquitoes that may be more vigorous and have a different disease-carrying potential. Therefore Public safety concerns must be respected and this experimental release must not be allowed to go ahead. It is also not known what impacts cross-breeding between GM mosquitoes and native mosquitoes may have on their ability to transmit diseases." (J. Smith 0095 p. 1)

GeneWatch UK (0335) and the Center for Food Safety (0344) pointed out that:

> "For comparison, in the UK, Oxitec has been prevented from releasing a GE diamondback moth (an agricultural pest) because of concerns about the use of a North American background strain, which is subject to controls under plant pest control regulations."[24] (GeneWatch UK 0335 p. 10, Center for Food Safety 0344 p. 12)

ii. Comments rebutting concerns about introgression resulting in hybrid vigor and disease transmission

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) indicated that Oxitec Ltd., would like to address a few salient topics raised in comment and thus was providing additional necessary context, information and technical details to the docket.

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) stated that:

> "Introgression into the wild mosquito population of natural mosquito genes present in OX5034 is expected to occur, and these genes are also expected to disappear from the environment over time. The natural background genetics of OX5034 were selected to ensure that OX5034 is susceptible to commonly-used insecticides, meaning that introgression of these genes into the wild population has the potential to help make the wild population less resistant to insecticides used to control mosquitoes." (N. Rose 0341 p. 7)

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) added that:

> "The natural genes passed on by the few surviving OX513A mosquitoes died out in treated areas after releases stopped;" (N. Rose 0341 p. 6)

[24] HSE (2011) Letter to Oxitec. 5 December 2011. Obtained by GeneWatch UK as the result of a Freedom of Information request.

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) also stated that:

> " . . , if any effect were to occur as a result of OX513A background genetics being introgressed into the local population, the effect would be expected to be beneficial, as introgression of insecticide-susceptible alleles would be expected to occur, restoring the effectiveness of insecticides against a local population that may have developed resistance." (N. Rose 0341 p. 7)

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) responding to the Evans et al[NOTEREF _Ref30598723 \h * MERGEFORMAT] statement that "introgression may introduce other relevant genes such as for pesticide resistance" stated that:

> " . . . OX513A is susceptible to standard insecticides (for example, pyrethroids and organophosphates) used for mosquito control. . . . (Carvalho et al., 2015)[25] . . . . (Patil et al., 2018)[26]." (N. Rose 0341 p. 6)

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) also stated that:

> "Natural genes carried by Oxitec mosquitoes do not confer increased capacity to transmit disease nor resistance to commonly used insecticides;" (N. Rose 0341 p. 3)

N. Rose, Head of Regulatory Science, Oxitec, Ltd., (0341) responding to the Evans et al[NOTEREF _Ref30598723 \h * MERGEFORMAT] statement that it is not known "what impacts introgression from a transgenic strain of Ae. aegypti has on traits of importance to disease control and transmission" stated that there is an:

> " . . . extensive body of literature that demonstrates that many of the factors most likely to affect vector competence are not genetic, but environmental, relating to the mosquito's microbiome and immune response, and relating to the genetics of the virus rather than the vector (Tabachnick, 2013[27]; Palmer, Varghese & Van Rij, 2018[28]; Souza-Neto, Powell & Bonizzoni, 2019[29])." (N. Rose 0341 p. 5)

---

[25] Carvalho DO., McKemey AR., Garziera L., Lacroix R., Donnelly CA., Alphey L., Malavasi A., Capurro ML. 2015. Suppression of a Field Population of Aedes aegypti in Brazil by Sustained Release of Transgenic Male Mosquitoes. *PLoS Negl Trop Dis* 9:e0003864. DOI: 10:1371/journal.pntd.0003864.

[26] Patil PB., Gorman KJ., Dasgupta SK., Reddy KVS., Barwale SR., Zehr UB. 2018. Self-Limiting OX513A Aedes aegypti Demonstrate Full Susceptibility to Currently Used Insecticidal Chemistries as Compared to Indian Wild-Type Aedes aegypti. *Psyche (New York)* 2018. DOI: 10.1155/2018/7814643.

[27] Tabachnick WJ. 2013. Nature, nurture and evolution of intra-species variation in mosquito arbovirus transmission competence. *International Journal of Environmental Research and Public Health* 10:249–277. DOI: 10.3390/ijerph10010249.

[28] Palmer WH., Varghese FS., Van Rij RP. 2018. Natural variation in resistance to virus infection in dipteran insects. *Viruses* 10:118. DOI: 10.3390/v10030118.

[29] Souza-Neto JA., Powell JR., Bonizzoni M. 2019. Aedes aegypti vector competence studies: A review. *Infection, Genetics and Evolution* 67:191–209. DOI: 10.1016/j.meegid.2018.11.009.

2. Comments Raising Questions About Potential Consequences of the tTAV-OX5034 and DsRed2-OX5034 Genes Introgressing into the Local Wild Aedes aegypti Mosquito Population

Friends of the Earth (0342) expressed concern about the genes engineered into OX5034 potentially introgressing into the local wild Aedes aegypti population. Friends of the Earth (0342) stated that:

> "According to the new application, Oxitec's male OX5034 GE mosquitoes are 'female-killing', which means that the GE males would mate with wild female mosquitoes. The female offspring would theoretically die, and the genetically engineered male OX5034 mosquitoes would theoretically survive into adulthood. This means that the OX5034 mosquitoes would increase the spread of the genetically engineered material into wild mosquito populations over several generations. However, there is no information or data about whether the spread of these genes would be beneficial to the environment or public health." (Friends of the Earth 0342 p. 2)

3. Comment on the Potential for Changes in OX5034 Engineered Genes from the Original Intended Insertion

Florida Keys Environmental Coalition (0331) expressed concern about the possibility of evolutionary changes affecting the genetic material engineered into OX5034. To address this concern the Florida Keys Environmental Coalition (0331) requested that OX5034 be vetted by:

> "Lab trials for multigenerational survivability of genetic modifications (Oxitec published results are from computer models) and the outcome of evolutionary changes to manmade mutations, and verification of characteristics of any hybridized species. These long-term studies are based on expected mutations Mendelian genetic heredity, where errors occur causing evolutionary change. Given that the DNA of this species has been perturbed then evolution may, or may not, be more likely over a shorter time frame, due to instability caused by intended and unintended unnatural DNA sequences. Observation is warranted." (Florida Keys Environmental Coalition 0331 p. 3-4)

The Florida Keys Environmental Coalition also pointed out that:

> " . . .there is no reason that the EPA should not seek an Environmental Impact Statement level investigation into many of the unknowns, including of[f]-site unintended mutation caused by imprecise RNA transcripts. These assays are now much quicker and much less expensive, so standardizing these as part of a GM Species standardized regiment and evaluation is now practical and advisable." (Florida Keys Environmental Coalition 0331 p. 3) [Typographical error in the original]

The Florida Keys Environmental Coalition (0331) also stated that the following should be done prior to any open field release program in the US:

> "Mosquitoes should be evaluated with uncharacterized unintended mutations through full DNA sequencing." (Florida Keys Environmental Coalition 0331 p. 3)

4. Calls for Additional Testing

The Center for Food Safety (0344) and GeneWatch UK (0335) stating that there "are no published peer-reviewed paper for Oxitec's GE Aedes aegypti OX5034 mosquitoes" indicated that necessary tests included:

> "A full, published investigation into the reported survival of hybrid GE mosquitoes in Brazil, including a specific investigation of the recent open release trials of OX5034 GE mosquitoes. This study should include detailed analysis of any hybrid mosquitoes for disease transmission properties."
> (GeneWatch UK 0335 p. 15-16)

GeneWatch UK (0335) stated that:

> "Very limited information regarding the newer OX5034 strain has been provided by the applicant in a published letter to the EPA[30]. The main substantive difference, compared to the earlier OX513A strain, is that the genetically engineered killing mechanism in OX5034 is intended to kill the female GE mosquitoes only, with GE males surviving for multiple generations. Although there are some important differences between the OX513A strain and the 2nd generation OX5034 strain, many of the issues raised regarding the 1st generation releases remain of concern and have not been addressed." (GeneWatch UK 0335 p. 1)

Friends of the Earth (0342) specified that:

> " . . . there is insufficient data about the sterility of the OX5034 mosquitoes, about the sites proposed for release in either Florida or Texas, and about Oxitec's proposed experimental program." (Friends of the Earth 0342 p. 1)

C. Comments Questioning Whether Adult OX5034 Female Mosquitoes or Their Offspring Females Expressing OX5034's Engineered Genes Might Occur in the Test Areas

Some commenters (Friends of the Earth 0342, GeneWatch UK 0335, Center for Food Safety 0344) expressed concern that little information is publicly available on whether Aedes aegypti

[30] Description of OX5034 Aedes aegypti Mosquito, including Active and Inert Ingredients. https://www.regulations.gov/docket?D=EPA-HQ-OPP-2019-0274

females carrying OX5034 genes, i.e., mosquitoes that bite, might at some point be in the test areas.

Anonymous (0329) stated that:

> "Oxitec/Intrexon plans to potentially release billions of mosquitoes and it is unclear [h]ow many of them will be biting and disease spreading females." (Anonymous 0329 p. 1 referencing: [HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313"]) [Typographical error in original]

Friends of the Earth (0342) commented that:

> "There is also no publicly accessible information about the likelihood that the female OX5034 mosquitoes could survive into adulthood, particularly in the presence of tetracycline. Similar to previous applications, Oxitec's claim that no biting GE females will survive in the environment is unsubstantiated. There needs to be an EIS with quantitative data about the effectiveness of the OX5034 mosquito's engineered "female-killing" trait, and the public should not be asked to rely on Oxitec's claims." (Friends of the Earth 0342 p. 2)

GeneWatch UK (0335) stated that:

> "It is also notable that no public information has been provided in the Docket or elsewhere relating to the survival rates of GE females to adulthood, in the presence or absence of sources of tetracycline: this makes it impossible to assess Oxitec's claim that no biting GE females will be released or survive to adulthood." (GeneWatch UK 0335 p.2)

Some commenters invoked past experience to support their concerns. GeneWatch UK (0335) and the Center for Food Safety (0344) stated that "Oxitec aims to release only male GE mosquitoes, however in practice large numbers of female GE mosquitoes – which may bite and transmit disease - have been released during past experiments with Oxitec's OX513A GE mosquitoes." (GeneWatch UK 0335 p. 5)

GeneWatch UK (0335) added that while Oxitec now states that "its new OX5034 strain will avoid this problem because it provides "*genetic separation to 100% males*"[NOTEREF _Ref26963493 \h * MERGEFORMAT]:

> " . . , Oxitec has provided no evidence that the female-killing mechanism engineered into the OX5034 strain is 100% effective. It is essential that such evidence is published and made available for independent scrutiny and consultation in order to assess the risk of release of female GE mosquitoes in the proposed experiments." (GeneWatch UK 0335 p. 5) [Emphasis in original]

Friends of the Earth (0342) pointed out that while "Oxitec's application states that female offspring of the OX5034 mosquitoes are expected to die before adulthood and therefore people won't be exposed to biting female mosquitoes":

> "Oxitec has not provided evidence for this claim[31]. Previously, Oxitec made claims about mosquito sterility, but a recent study from the Powell lab at Yale University confirmed that Oxitec claims that the mosquitoes were sterile were not true, and that some of the offspring of Oxitec's genetically engineered mosquitoes survived into adulthood.[NOTEREF _Ref30598723 \h * MERGEFORMAT]Genetic material was spread into wild populations of mosquitoes, and the direct and indirect environmental and health impacts of the new mosquitoes carrying novel genetics were concerning." (Friends of the Earth 0342 p. 4)

In further support of their concerns, GeneWatch UK (0335) and Center for Food Safety (0344) pointed to past experience with Oxitec's OX513A mosquito strain:

> "Oxitec used a mechanical method to sort its OX513A GE mosquitoes by size, with the aim of releasing mainly male mosquitoes, which do not bite. In 2014, Oxitec published a number of figures on the number of biting female GE mosquitoes that are inadvertently released[32],[33]. In practice, these criteria were often exceeded. For example, checks by the Mosquito Research and Control Unit (MRCU) in the Cayman Islands on one production batch on May 12th 2017 revealed 9 females in one release pot of 500 (1.8%), nine times the agreed level.[34] The Cayman Islands' report also shows significant increases (spikes) in adult female mosquito numbers (green line in Figure 1B) in the release area 5 to 7 weeks after the releases begin, and again 7 to 8 weeks after the releases are increased.[NOTEREF _Ref27143590 \h * MERGEFORMAT] These spikes in the adult female population exceed 150% of the comparator population, but their true extent is not shown as the peaks are cut off on the graph. These female GE mosquitoes pose a risk to the public because they can bite and transmit disease. Emails released as a result of a Freedom of Information (FOI) request in the Cayman Islands highlight "*a significant increase in the number of female mosquitoes collected in the treatment area*", rather than a decrease, which is thought to be due to the accidental release of GE female mosquitoes.[35] The emails

---

[31] Public comments from GeneWatch UK. http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/GeneWatch_EPA_Oxitec_consul19_fin.pdf

[32] Carvalho DO, Nimmo D, Naish N, et al. (2014) Mass Production of Genetically Modified *Aedes aegypti* for Field Releases in Brazil. *Journal of Visualized Experiments*. (83). doi:10.3791/3579. http://www.jove.com/video/3579/mass-production-genetically-modified-aedes-aegypti-for-field-releases

[33] Harris AF et al. (2011) Field performance of engineered male mosquitoes. *Nat. Biotech.*, 29(11), 1034-1037.

[34] Annual report MRCU - June 2017. Friendly *Aedes aegypti* project in West Bay. http://www.genewatch.org//uploads/f03c6d66a9b354535738483c1c3d49e4/MRCU_annual_report___Oxitec_project___June_2017.pdf

[35] Oxitec Project: Results to date. Attachment to email from MRCU scientist to Chief Officer, Ministry of Health, Environment, Culture and Housing (HECH), 4 April 2017. Communications between MRCU and Ministry. Released as a result of a Freedom of Information (FOI) request. 3 April 2018.

> reveal a high level of concern about the inadvertent release of GE female mosquitoes, from the Mosquito Research and Control Unit (MRCU) scientist with access to the data."[36] (Center for Food Safety 0344 p. 7) [Emphasis in the original]

Anonymous 0226 expressed the opinion that:

> " . . the genetic trait is passed on to both the male and female offspring that are produced when the released GM male mosquitoes mate with wild females. Some of these GM female larvae will also survive to adulthood." (Anonymous 0226 p. 9-10)

Florida Keys Environmental Coalition (0331) stated that:

> "Quantified evaluation of any potential female hatching needs to be monitored by observing a statistically significant number of eggs reaching fruition." (Florida Keys Environmental Coalition 0331 p. 3)

1. Comments Suggesting Mechanisms Through Which Biting Aedes aegypti Female Mosquitoes Carrying OX5034 Genes Might be Present in the Test Area

Comments offered suggested mechanisms through which biting Aedes aegypti females carrying tTAV-OX5034 and/or DsRed2-OX5034 genes might be present in the test area. Comments on routes through which this might occur include: sufficient tetracycline in the environment to allow tetracycline-dependent females to mature to adults; failure of the tetracycline-dependency gene in OX5034 to have complete penetrance; resistance to the killing mechanism evolving in the mosquito; behavioral adaptation by the mosquito to ensure survival of offspring; contamination of OX5034 adult male releases by adult OX5034 biting females.

The Center for Food Safety (0344) and GeneWatch UK (0335) pointed out that if any of these mechanisms allow female Aedes aegypti larvae to mature to adults:

> "Eggs may survive for several months when dried out on the inner walls of containers and may be transported elsewhere.[37] Any assessment therefore needs to consider the potential global transport of such eggs, and not be limited to considering the lifespan of adults and dispersal through adult flying." (Center for Food Safety 0344 p. 8; GeneWatch UK 0335 p. 7)

Anonymous (0329) cautioned that "Once released, GM mosquitoes cannot be contained":

> "Aedes aegypti can travel up to 3 miles in certain conditions and even to other countries

---

http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/Communication_between_MRCU_and_Ministry_1.pdf

[36] Email from MRCU scientist to MRCU Director and other staff, 11 August 2017. Communications between MRCU staff. Released as a result of a Freedom of Information (FOI) request. 3 April 2018. http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/Communication_between_MRCU_staff.pdf

[37] CDC (undated) Dengue: Entomology & Ecology http://www.cdc.gov/dengue/entomologyEcology/index.html

> if they enter a vehicle, placing them well outside of any city or county they are released in." (Anonymous 0329 p. 2 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

Anonymous (0329) also noted that:

> "Aedes aegypti are able to survive in cold weather.
> "Aedes aegypti are able to breed in water with high levels of salinity.
> "Aedes aegypti eggs can remain viable for up to 450 days.
> (Anonymous 0329 p. 2 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

Anonymous (0329) added that:

> "GM mosquitoes could cause a huge increase in Aedes aegypti population if the lethality trait fails." (Anonymous 0329 p. 2 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

2. Comments Questioning Whether Sufficient Tetracycline Occurs in the Test Environment to Allow Tetracycline-Dependent Female Aedes aegypti Mosquitoes to Mature to Adults

Some commenters (Friends of the Earth 0342, Center for Food Safety 0344, GeneWatch UK 0335) suggested that consideration be given to the possibility that there may be sufficient tetracycline present in the testing environments to influence the ability of tetracycline-dependent female Aedes aegypti to mature to biting adults.

The Center for Food Safety (0344) and GeneWatch UK (0335) commented that:

> "In its 2004 report, the National Research Council's (NRC's) Committee on the Biological Confinement of Genetically Engineered Organisms (GEOs) states that biological confinement (bioconfinement) includes the use of biological barriers, such as induced sterilization, that prevent GEOs or transgenes from surviving or reproducing in the natural environment (page 15).[38] The report emphasises the importance of considering the large scale at which bioconfined organisms could be released and the possibility that even carefully planned, integrated bioconfinement methods could fail. It concludes that research is needed to characterize potential ecological consequences of bioconfinement methods and to develop methods and protocols for assessing environmental effects should confinement fail (page 12)." (Center for Food Safety 0344 p. 8; GeneWatch UK 0335 p. 7)

The Center for Food Safety (0344) and GeneWatch UK (0335) pointed out that:

---

[38] NRC (2004) Biological Confinement of Genetically Engineered Organisms. Committee on the Biological Confinement of Genetically Engineered Organisms, National Research Council. ISBN: 0-309-52778-3, 284 pages. http://www.nap.edu/catalog.php?record_id=10880

> " . . . the lethality trait is conditional: the company uses the common antibiotic tetracycline as a chemical switch to turn off the killing mechanism, allowing the insects to be bred in the laboratory. This mechanism can therefore fail if the GE mosquitoes encounter high enough levels of tetracycline in the environment." (Center for Food Safety 0344 p. 9; GeneWatch UK 0335 p. 7)

Friends of the Earth (0342) pointed out that:

> "There is no data to confirm the survival rate of the GE females in the environment, both with or without the presence of tetracycline." (Friends of the Earth 0342 p.3-4)

Some commenters (Friends of the Earth 0342, Center for Food Safety 0344) stated that tetracycline is a commonly used antibiotic and it is possible that mosquito larvae might encounter sufficiently high enough concentrations of the antibiotic to allow the larvae to mature to adults. Commenters indicated 3 routes by which sufficient concentrations of tetracycline might become available to allow Aedes aegypti larvae to mature to adult mosquitoes: through agricultural production use, in sewage, or in food production and animal husbandry.

i. Agricultural production

Some comments offered suggestions on agricultural processes that might provide routes through which tetracycline could be present in the test environment.

For example, Friends of the Earth (0342) commented that:

> " . . , tetracycline is a common antibiotic used in agriculture production, and Florida citrus growers use significant amounts of tetracyclines (oxytetracycline) on agricultural lands as a pesticide in efforts to control the bacteria responsible for the Citrus Greening disease. The significant presence of tetracycline in the environment may obviate the lethal trait in the GE mosquitoes and their offspring could survive and continue to breed." (Friends of the Earth 0342 p.3-4)

The Center for Food Safety (0344) also pointed out that:

> "Oxytetracycline is being tested as a control for citrus greening disease and it could be sprayed in the future on citrus in Monroe County, FL and Harris County, TX."[39],[40] (Center for Food Safety 0344 p. 9)

ii. Sewage

---

[39] Kyselkova, M., et al. (2013), Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. *Chemosphere*. **93**(10): 2413-8.

[40] Ho, Y.B., et al. (2012) Simultaneous determination of veterinary antibiotics and hormone in broiler manure, soil and manure compost by liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. **1262**: 160-8.

Some comments offered suggestions on alternative routes through which tetracycline might be present in the test environment.

Friends of the Earth (0342) stated that:

> " . . , tetracycline is also a prevalent compound found in sewage, due to contamination from agricultural run-off and consumer disposal, for example. *Aedes aegypti* have been found to breed in sewage treatment plants, septic tanks, and cesspits in the Florida Keys.[41] The possible widespread application and presence of tetracycline in the environment could significantly undermine the efficacy of GE mosquitoes to reduce overall mosquito populations. The 'female-killing' trait might fail if the mosquitoes are in contact with sufficiently high levels of tetracycline." (Friends of the Earth 0342 p. 4)

The Center for Food Safety (0344) added additional support for this comment pointing out that:

> "In the case of the OX513A strain, Oxitec claimed that an increased survival rate due to tetracycline contamination would not happen in the wild because the GE larvae would breed only in clean water. However, a number of studies have found that *Aedes aegypti* mosquitoes can breed in septic tanks where there can be high levels of contamination with antibiotics such as tetracycline.[NOTEREF _Ref26976961 \h * MERGEFORMAT], [42], [43], [44], [45]. A 2004 study found that sewage treatment plants, septic tanks, and cesspits were larval development sites for *Aedes aegypti* in the Florida Keys.[NOTEREF _Ref26976961 \h * MERGEFORMAT] In 2004, there were more than 36,000 septic systems and 5,000 to 10,000 cesspits in Florida."[46] (Center for Food Safety 0344 p. 9) [Duplicative references edited out]

iii. Food, food waste and animal husbandry

GeneWatch UK (0335) added that:

> *"Ae. aegypti* also commonly live in areas where discarded food is likely to contain meat contaminated with tetracycline; cat food would have sufficient amounts of tetracycline

---

[41] Hribar, L. J., Vlach, J. J., Demay, D. J., James, S. S., Fahey, J. S., and Fussell, E. M. 2004. Mosquito larvae (Culicidae) and other Diptera associated with containers, storm drains, and sewage treatment plants in the Florida Keys, Monroe County, Florida. Florida Entomol. 87: 199–203.

[42] Irving-Bell RJ, Okoli EI, Diyelong DY, Lyimo EO, Onyia OC (1987). Septic tank mosquitoes: competition between species in central Nigeria. *Medical and Veterinary Entomology*, 1, 243-250.

[43] Barrera R, Amador M, Diaz A, Smit J, Munoz-Jordan JL, Rosario Y (2008). Unusual productivity of *Aedes aegypti* in septic tanks and its implications for dengue control. *Medical and Veterinary Entomology*, 22, 62-69.

[44] Beserra EB, Fernandes CRM, de Sousa JT, de Freitas EM, Santos KD (2010). Efeito da qualidade da água no ciclo de vida e na atração para oviposição de *Aedes aegypti* (L.) (Diptera: Culicidae). *Neotropical Entomology*, 39, 1016-1023.

[45] Burke R, Barrera R, Lewis M, Kluchinsky T, Claborn D (2010). Septic tanks as larval habitats for the mosquitoes *Aedes aegypti* and *Culex quinquefasciatus* in Playa-Playita, Puerto Rico. *Medical and Veterinary Entomology*, 24, 117-123.

[46] Griffin DW (2004) Florida's Geology Makes Wastewater Disposal a Potential Threat to Ecosystem Health in the Florida Keys. Sound Waves. October 2004. http://soundwaves.usgs.gov/2004/10/research.html

> to keep the mosquitoes alive. Oxitec uses a diet supplemented with 30 µg/ml of the tetracycline to breed its OX513A mosquitoes in the lab: again, figures are not available for the OX5034 strain. The tetracycline derivatives oxytetracycline (OTC) and doxycycline (DOX, used to prevent malaria) could also allow the GE mosquitoes to breed. Oxytetracycline can be found at concentrations above 500 µg/g in animal manure and doxycycline at up to 78516.1 µg/kg dry weight in broiler manure, which may be sufficient to inactivate the killing mechanism." (GeneWatch UK 0335 p. 7)

The Center for Food Safety (0344) added that:

> "When OX513A GE mosquitoes were fed cat food containing industrially farmed chicken, which contains the antibiotic tetracycline, the survival rate increased to 15-18%. Oxitec . . . admitted to an 18% survival rate of larvae fed on cat food in a published paper.[47] In the case of the OX5034 strain, no information has been provided whatsoever on the impacts of tetracycline on the likely survival rates of GE female mosquitoes." (Center for Food Safety 0344 p. 9)

iv. Comments rebutting the possibility that there may be sufficient tetracycline in the environment to allow female Aedes aegypti carrying the tTAV-0534 gene to mature to adults.

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) stated that:

> **"Environmental levels of tetracyclines high enough to help female OX5034 mosquitoes survive have never been recorded in the USA in potential *Aedes aegypti* breeding sites**, based on a comprehensive survey of the peer-reviewed literature (Meyer et al., 2000[48]; Lindsey, Meyer & Thurman, 2001[49]; Campagnolo et al., 2002[50]; Yang & Carlson,

---

[47] Massonnet-Bruneel B, Corre-Catelin N, Lacroix R, et al. (2013) Fitness of Transgenic Mosquito Aedes aegypti Males Carrying a Dominant Lethal Genetic System. *PLoS ONE*. **8**(5):e62711. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3653897/

[48] Meyer MT., Bumgarner JE., Varns JL., Daughtridge J V., Thurman EM., Hostetler KA. 2000. Use of radioimmunoassay as a screen for antibiotics in confined animal feeding operations and confirmation by liquid chromatography/mass spectrometry. In: *Science of the Total Environment*. 181–187. DOI: 10.1016/S0048-9697(99)00541-0.

[49] Lindsey ME., Meyer TM., Thurman EM. 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Analytical chemistry* 73:4640–6.

[50] Campagnolo ER., Johnson KR., Karpati A., Rubin CS., Kolpin DW., Meyer MT., Esteban JE., Currier RW., Smith K., Thu KM., McGeehin M. 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *Science of the Total Environment* 299:89–95. DOI: 10.1016/S0048-9697(02)00233-4.

2003[51]; Yang, Cha & Carlson, 2004[52], 2005[53]; Kim et al., 2005[54]; Karthikeyan & Meyer, 2006[55]; Batt, Bruce & Aga, 2006[56]; MacKie et al., 2006[57]; Batt, Kim & Aga, 2007[58]; Dolliver & Gupta, 2008b[59],a[60]; Haggard & Bartsch, 2009[61]; Kulkarni et al., 2017[62]). The highest reported concentrations of environmental tetracyclines would be insufficient to allow survival of any female hemizygous OX5034 life-stages. The testing of antibiotic concentrations found in the environment is frequently associated with the efficacy of waste water treatment plants at removing antibiotics from waste water. Samples are taken from influent and effluent, and from rivers downstream of treatment plants. Antibiotic concentrations are also frequently tested in hog lagoons, which are anaerobic lagoons used to treat animal waste from farming pigs or other livestock. These are not typical breeding locations for *Ae. aegypti* larvae. *Ae. aegypti* is commonly referred to as a 'container breeding mosquito' as its preferred breeding sites include flower vases, tires, tree holes, etc. They are found in clean, still water, not flowing river systems and are rarely found in collections of water in the ground such as borrow-pits or earth drains

---

[51] Yang S., Carlson K. 2003. Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Research* 37:4645–4656. DOI: 10.1016/S0043-1354(03)00399-3.

[52] Yang S., Cha J., Carlson K. 2004. Quantitative determination of trace concentrations of tetracycline and sulfonamide antibiotics in surface water using solid-phase extraction and liquid chromatography/ion trap tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* 18:2131–2145. DOI: 10.1002/rcm.1598.

[53] Yang S., Cha J., Carlson K. 2005. Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry. *J Chromatogr A* 1097:40–53. DOI: 10.1016/j.chroma.2005.08.027.

[54] Kim S., Eichhorn P., Jensen JN., Weber AS., Aga DS. 2005. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environmental Science and Technology* 39:5816–5823. DOI: 10.1021/es050006u.

[55] Karthikeyan KGG., Meyer MT. 2006. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, 11 USA. *Sci Total Environ* 361:196–207. DOI: 10.1016/j.scitotenv.2005.06.030.

[56] Batt AL., Bruce IB., Aga DS. 2006. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environmental Pollution* 142:295–302. DOI: 10.1016/j.envpol.2005.10.010.

[57] MacKie RI., Koike S., Krapac I., Chee-Sanford J., Maxwell S., Aminov RI. 2006. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. *Animal Biotechnology* 17:157–176. DOI: 10.1080/10495390600956953.

[58] Batt AL., Kim S., Aga DS. 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* 68:428–435. DOI: 10.1016/j.chemosphere.2007.01.008.

[59] Dolliver HAS., Gupta SC. 2008b. Antibiotic Losses from Unprotected Manure Stockpiles. *Journal of Environment Quality* 37:1238. DOI: 10.2134/jeq2007.0391.

[60] Dolliver H., Gupta S. 2008a. Antibiotic Losses in Leaching and Surface Runoff from Manure-Amended Agricultural Land. *Journal of Environment Quality* 37:1227. DOI: 10.2134/jeq2007.0392.

[61] Haggard BE., Bartsch LD. 2009. Net Changes in Antibiotic Concentrations Downstream from an Effluent Discharge. *Journal of Environment Quality* 38:343. DOI: 10.2134/jeq2007.0540.

[62] Kulkarni P., Olson ND., Raspanti GA., Goldstein RER., Gibbs SG., Sapkota A., Sapkota AR. 2017. Antibiotic concentrations decrease during wastewater treatment but persist at low levels in reclaimed water. *International Journal of Environmental Research and Public Health* 14:668. DOI: 10.3390/ijerph14060668.

(Christophers, 1960[63]; Morrison et al., 2006[64]; Dieng et al., 2012[65]). Some reports have suggested that *Ae. aegypti* can breed in septic tanks (Barrera et al., 2008[66]; Mackay et al., 2009[67]) but this tends to be in the clear water at the top of the tank whereas tetracycline and their analogues tend to bind to the sediment which collects at the bottom (Brown et al., 2006[68]; Watkinson et al., 2009[69]). Therefore, the concentrations that resulted in functional female phenotypic rescue in this study are very unlikely to be found in typical breeding sites of *Ae. aegypti* (Curtis et al., 2015[70]), therefore the potential for the efficacy of a control program using OX5034 to be compromised by the current reported levels of environmental tetracycline and its analogues is negligible." (N. Rose 0341 p.8-9) [Emphasis in original]

3. Comments Positing the Possibility of Failure of the Tetracycline-Dependency Gene in OX5034 to Have Complete Penetrance

Some commenters (GeneWatch UK 0335, Center for Food Safety 0344) suggested that the possibility that the tetracycline-dependency gene in OX5034 might not have complete penetrance be evaluated as this could lead to unintentional survival of biting Aedes aegypti females carrying the OX5034 genes.

GeneWatch UK (0335) and the Center for Food Safety (0344) stated that:

"Oxitec's approach to reducing the reproductive capacity of its GE mosquitoes has a number of major weaknesses. Firstly, the killing trait may not be fully penetrant (meaning not all the GE insects will die) and is late-acting (meaning the insects are not sterile, but mostly die at the late larval stage). In the case of its OX513A strain, Oxitec

---

[63] Christophers R. 1960. *Aedes aegypti (L.) The Yellow Fever Mosquito: Its LIfe History, Bionomics and Structure*. Cambridge University Press.

[64] Morrison AC., Sihuincha M., Stancil JD., Zamora E., Astete H., Olson JG., Vidal-Ore C., Scott TW. 2006. Aedes aegypti (Diptera: Culicidae) production from non-residential sites in the Amazonian city of Iquitos, Peru. *Ann Trop Med Parasitol* 100 Suppl:S73–S86. DOI: 10.1179/136485906X105534.

[65] Dieng H., Saifur RG., Ahmad AH., Salmah MR., Aziz AT., Satho T., Miake F., Jaal Z., Abubakar S., Morales RE. 2012. Unusual developing sites of dengue vectors and potential epidemiological implications. *Asian Pac J Trop Biomed* 2:228–232. DOI: 10.1016/S2221-1691(12)60047-1.

[66] Barrera R et al., Amador M., Diaz A., Smith J., Munoz-Jordan JL., Rosario Y. 2008. Unusual productivity of Aedes aegypti in septic tanks and its implications for dengue control. *Med Vet Entomol* 22:62–69. DOI: 10.1111/j.1365-2915.2008.00720.x.

[67] Mackay AJ., Amador M., Diaz A., Smith J., Barrera R. 2009. Dynamics of Aedes aegypti and Culex quinquefasciatus in septic tanks. *J Am Mosq Control Assoc* 25:409–416. DOI: 10.2987/09-5888.1.

[68] Brown KD., Kulis J., Thomson B., Chapman TH., Mawhinney DB. 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent,municipal wastewater, and the Rio Grande in New Mexico. *Science of the Total Environment* 366:772–783. DOI: 10.1016/j.scitotenv.2005.10.007.

[69] Watkinson AJ., Murby EJ., Kolpin DW., Costanzo SD. 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Science of the Total Environment* 407:2711–2723. DOI: http://dx.doi.org/10.1016/j.scitotenv.2008.11.059.

[70] Curtis Z., Matzen K., Oviedo MN., Nimmo D., Gray P., Winskill P., Locatelli MAF., Jardim WF., Warner S., Alphey L., Beech C. 2015. Assessment of the impact of potential tetracycline exposure on the phenotype of Aedes aegypti OX513A: Implications for field use. *PLoS Neglected Tropical Diseases* 9. DOI: 10.1371/journal.pntd.0003999.

> published evidence that 3 to 4% of these GE mosquitoes unintentionally survived to adulthood [71]: however, no information has been provided on the penetrance of the female-killing trait in OX5034. This means it is impossible to assess how many GE female mosquitoes might survive to adulthood." (GeneWatch UK 0335 p.7; Center for Food Safety 0344 p. 8-9)

Friends of the Earth (0342) noted that:

> " . . . with the OX513A mosquitoes, 3 to 4 percent of Oxitec's mosquitoes survived into adulthood in the lab in the absence of tetracycline despite carrying the lethal gene[NOTEREF _Ref29570949 \h * MERGEFORMAT]. There is no data to confirm the survival rate of the GE females in the environment, both with or without the presence of tetracycline." (Friends of the Earth 0342 p. 3-4) [Duplicative references edited out]

Anonymous 0226 stated that:

> "In the laboratory, 3% of the offspring of Oxitec's GM mosquitoes survive to adulthood, even in the absence of the antibiotic tetracycline.[NOTEREF _Ref29570949 \h * MERGEFORMAT] When GM mosquitoes were fed cat food containing industrially farmed chicken, which contains the antibiotic tetracycline, the survival rate increased to 15-18%. Oxitec . . . admitted to an 18% survival rate in a published paper."[NOTEREF _Ref30509932 \h * MERGEFORMAT] (Anonymous 0226 p. 11)

4. Comments Positing that Resistance to the Killing Trait Might Evolve in the Mosquito

Some commenters (Center for Food Safety 0344, GeneWatch UK 0335) pointed out that A. aegypti mosquitoes might evolve resistance to the tTAV-OX5034 trait.

The Center for Food Safety (0344) stated that:

> "In addition, it is possible that the mosquitoes could develop a resistance to the lethality trait, which could lead to biting GE females being released into the environment. This further accentuates the EPA's need for a complete EIS and more thorough examination of unintended consequences before allowing Oxitec's application to be considered." (Center for Food Safety 0344 p. 9)

The Center for Food Safety (0344) noted that:

> ". . , resistance to the killing mechanism could evolve in the GE mosquito factory or in the environment. (Center for Food Safety 0344 p. 9)

---

[71] Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ et al. (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology*, **5**: 11. http://www.biomedcentral.com/1741-7007/5/11

GeneWatch UK (0335) stated that:

> "The percentage of surviving GE mosquitoes could also increase if resistance to the genetic killing mechanism evolves over time.[72] In comparison, the traditional Sterile Insect Technique (SIT), used to control some pests, results in multiple chromosome breaks when the insects are exposed to radiation, severely limiting any potential for resistance to evolve during the production process. In contrast, any genetic or molecular event that allows the GE mosquitoes to survive and breed successfully could be rapidly selected for during mass production.[73] Increased survival rates would reduce the effectiveness of any population suppression effect over time, increase the number of biting GE females, and potentially allow the GE mosquitoes to establish in the wild." (GeneWatch UK 0335 p. 7-8)

5. Comments Positing that Mosquitoes Could Adapt Behaviorally to Ensure Survival of Offspring Carrying the tTAV-OX5034 Trait

One commenter (GeneWatch UK 0335) argued that the possibility of adaptive behavior ensuring the survival of offspring evolving in the Aedes mosquito population should be considered.

GeneWatch UK (0335) noted that:

> "In a conventional SIT programme in Japan, wild females appeared that were unreceptive to mating with irradiated males.[74] Therefore, adaptive behaviour in wild females to increase survival of their offspring, including avoiding GE males or seeking out tetracycline-contaminated sites to lay their eggs, must also be considered." (GeneWatch UK 0335 p. 7-8)

6. Comments Positing that Contamination of Adult Male OX5034 Mosquito Releases by Adult OX5034 Females Could Occur

Some commenters (Center for Food Safety 0344; GeneWatch UK 0335) expressed concern that releases of adult OX5034 males might be contaminated by adult OX5034 females.

The Center for Food Safety (0344) and GeneWatch UK (0335) stated that:

---

[72] Alphey N, Bonsall B, Alphey A (2011) Modeling resistance to genetic control of insects. *Journal of Theoretical Biology*, 270, 42-55.

[73] Robinson AS, Franz G, Atkinson PW (2004) Insect transgenesis and its potential role in agriculture and human health. *Insect Biochemistry and Molecular Biology*, **34**(2), 113–120.

[74] Hibino Y, Iwahashi O, 1991. Appearance of wild females unreceptive to sterilized males on Okinawa Is. in the eradication program of the melon fly, *Dacus cucurbitae* Coquillet (Diptera: Tephritidae). *Applied Entomology and Zoology*, **26**(2), 265–270.

> "Steps are also required to ensure that the GE mosquito line is not contaminated with potentially surviving females, or that other unexpected events do not occur. This has already been a major problem with during caged experiments using Oxitec's flightless female GE mosquitoes in Mexico. Quartz reports[NOTEREF _Ref27054321 \h * MERGEFORMAT]: "*However, during an experiment, one of the research partners found that some of the GM mosquitoes only had one copy of the gene rather than the two needed to pass on the trait consistently—meaning half of their female offspring could fly, and mate. The GM mosquito line was likely contaminated during an earlier experiment in Colorado; at some point, a wild mosquito probably sneaked into the GM mosquito insectary. The line returned to Oxitec in the UK before shipping to Mexico, said Luca Facchinelli, a medical entomologist at the University of Perugia, who managed the field site*". The GE mosquitoes to be released under the proposed permit are different: however, open release trials are premature in the absence of a full, published investigation into this incident, to establish whether or not contamination was the cause, and protocols to prevent further errors of this kind." (Center for Food Safety 0344 p. 7; GeneWatch UK 0335 p. 6) [Emphasis in original]

Anonymous 0226 stated that:

> "Emails released as a result of a Freedom of Information (FoI) request in the Cayman Islands highlight "a significant increase in the number of female mosquitoes collected in the treatment area, rather than a decrease, which is thought to be due to the accidental release of GM female mosquitoes.[NOTEREF _Ref27143347 \h * MERGEFORMAT] The emails reveal a high level of concern about the inadvertent release of GM female mosquitoes, from the MRCU scientist with access to the data.[NOTEREF _Ref27143456 \h * MERGEFORMAT] A 2017 report includes female adult mosquito numbers collected from traps in the published data.[NOTEREF _Ref27143590 \h * MERGEFORMAT] The graph shows significant increases (spikes) in adult female mosquito numbers in the release area five to seven weeks after the releases begin, and again seven to eight weeks after the releases were stepped up." (Anonymous 0226 p. 10)

7. Comments Calling for Additional Testing Before Releases are Permitted

Several commenters requested that additional testing be performed and independently replicated before releases are permitted.

The Center for Food Safety (0344) and GeneWatch UK (0335) stating that there "are no published peer-reviewed paper for Oxitec's GE Aedes aegypti OX5034 mosquitoes" indicated that necessary tests include:

> "• Independent verification that the new OX5034 strain provides Oxitec's claimed "*genetic separation to 100% males*": plus estimates of the numbers of GE biting female mosquitoes that may be released during the proposed experiments, or that may survive

> from subsequent generations, taking into account the potential to encounter tetracycline in the environment.
> • Studies of the potential of the GE mosquitoes to evolve resistance to the killing mechanism during mass breeding or following release, plus studies of the potential for wild females to evolve behavioural resistance.
> •Identification of potential sites where GE mosquitoes could encounter industrially farmed meat (e.g. discarded takeaways, pet food) and testing of tetracycline levels at these sites.
> •A full, published investigation into the reported survival of hybrid GE mosquitoes in Brazil, including a specific investigation of the recent open release trials of OX5034 GE mosquitoes.
> •A full, published investigation into the unexpected survival of female mosquitoes in Oxitec's experiments in Mexico." (Center for Food Safety 0344 p. 18)

The Center for Food Safety (0344) and GeneWatch UK (0335) added that necessary tests included:

> "Identification of relevant septic tanks and cess pits where mosquitoes may breed and testing of tetracycline levels in them." (GeneWatch UK 0335 p. 15-16)

Finally, GeneWatch UK (0335) and Center for Food Safety (0344) added that the necessary studies would include:

> "Full independent testing of the non-native strain proposed for release, for disease transmission traits for all relevant diseases and insecticide resistance for all relevant insecticides, plus contained studies addressing concerns about the potential 'hybrid vigour' of any hybrid strains." (GeneWatch UK 0335 p. 15; Center for Food Safety 0344 p. 18)

Friends of the Earth (0342) urged EPA to pay particular attention to assessments that look at:

> "•The risks of releasing biting females
> • Potential locations where significant levels of tetracycline may be present.
> • The risks associated with mosquitoes surviving into adulthood if tetracycline is present in the surrounding environment." (Friends of the Earth 0342 p. 7)

II. Human Health

Human health comments revolved around potential for: A. aegypti mosquitoes with greater competency to transmit viral pathogens, the proteins engineered into OX5034 to be toxic or allergenic, and use of OX5034 in the environment to spread, in microbial populations, resistance to the antibiotics used to grow OX5034.

A. Comments on Potential for Greater Competency

Some commenters (Friends of the Earth 0342, GeneWatch UK 0335, Anonymous 0329, T. Ritchie 0223) expressed concern that female Aedes aegypti mosquitoes associated with the EUP releases might vector disease, and that disease transmission might increase because of the testing. Commenters said the following should be considered: already infected females might be released during the testing, biting females carrying OX5034 genes could become infected post-release, hybrid vigor might lead to increased vector competency, and pathogenic viruses could evolve in response to OX5034.

1. Comments Raising Concerns that Infected Females Might be Released During Testing

Friends of the Earth (0342) stated that experience shows there could be a potential for biting female Aedes aegypti mosquitoes to be released during the testing:

> "Oxitec's initial Draft EA to the FDA about the OX513A mosquitoes acknowledges that it is inevitable that some biting female GE mosquitoes will be released. Similarly, GE female OX5034 mosquitoes, which can bite and transmit disease, could be released into the environment during the experiments. Biting females could already be infected with diseases . . . ." (Friends of the Earth 0342 p. 5)

GeneWatch UK (0335) went on to caution that "the possibility that the released GE mosquitoes are already infected with diseases also needs to be considered":

> "Oxitec's draft Environmental Assessment for its OX513A strain, as submitted to the FDA (page 31), stated that the horse blood it uses to feed the GE mosquitoes at its UK production facility is screened for equine infectious anemia (EIA) and equine viral arteritis (EVA) among other pathogens, to minimize the potential for contamination of the blood by virus, bacteria, or other pathogenic agents[75]. It also notes that the host range of *Aedes aegypti* and *Aedes albopictus* does not extend to the UK, so the risk of transmission of arbovirus such as dengue and chikungunya to these horses is negligible. However, the range of *Aedes albopictus* has been expanding in Europe and there have been warnings that this vector could reach the UK in future[76],[77]. The UK has several endemic mosquito species (mainly *Culex* species) that could potentially act as vectors for West Nile Virus in the future. It is also unclear what feed source Oxitec intends to use in its US rearing facilities. To reduce the risk that infected mosquitoes (potentially including some biting females) are released, a protocol for testing the GE mosquitoes for pathogenic agents should be introduced at the proposed rearing facilities. Up-to-date

---

[75] Oxitec (2016) Draft Environmental Assessment for Investigational Use of *Aedes aegypti* OX513A. https://www.fda.gov/downloads/animalveterinary/developmentapprovalprocess/geneticengineering/geneticallyengineeredanimals/ucm487377.pdf

[76] Mosquitoes 'could bring exotic diseases to UK'. BBC. 23rd March 2015. http://www.bbc.co.uk/news/health-31971996

[77] Medlock JM, Leach SA (2015) Effect of climate change on vector-borne disease risk in the UK. *The Lancet Infectious Diseases*, **15**(6), 721–730. http://doi.org/10.1016/S1473-3099(15)70091-5

> information regarding the feeding of the OX5034 strain also needs to be provided. (GeneWatch UK 0335 p. 6)

Friends of the Earth (0342) urged that EPA should ensure that there are testing protocols in place to prevent release of infected OX5034 mosquitoes:

> " . . . and the EPA should ensure that there is a stated protocol for testing the GE mosquitoes for pathogenic agents at the proposed rearing facilities." (Friends of the Earth 0342 p. 5)

2. Biting Females Carrying OX5034 Genes Could Become Infected Post-Release

GeneWatch UK (0335) reminded that:

> "Biting females may transmit disease even if they are disease-free on release (or at the time of birth in the environment), since they may encounter one of the diseases for which the *Aedes aegpti* mosquito is a vector (e.g. dengue, zika, chikungunya, yellow fever) by biting an infected person or animal, and spread that disease by subsequently biting an uninfected person or animal." (GeneWatch UK 0335 p. 6)

3. Hybrid Vigor Might Lead to Increased Vector Competency

Some commenters (L.M. Castro 0332, Anonymous 0329, T. Ritchie 0223) referring to the recent publication by Evans et al,[NOTEREF _Ref30598723 \h * MERGEFORMAT] expressed concern that introgression of the genes carried by released OX5034 mosquitoes might result in increases in the transmission of viruses carried by A. aegypti.

Quoting José Maria Gusman Ferraz, a researcher at Ecological Engineering Laboratory of Unicamp (the University of Campinas, a public research university in the state of São Paulo, Brazil), L.M. Castro (0332) noted that "there was gene exchange":

> " . . . resulting in hybrid insects, which usually have greater vigour and are more potent – yet there are no studies on these hybrids. Even less is known about the hybrid's efficiency in virus transmission, which may even be higher.[NOTEREF _Ref27149257 \h * MERGEFORMAT]"(L.M. Castro 0332 p. 1-2) [L.M. Castro (0332) noted that this was translated from the original Portuguese by GMWATCH[NOTEREF _Ref27149257 \h * MERGEFORMAT]]

Commenter Anonymous 0329 stated that:

> "The long term impact of the GMO genes in the wild population has not been studied and could cause the wild population to be more virulent, resulting in higher rates of mosquito borne disease." (Anonymous 0329 p. 1 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

T. Ritchie (0223) stated concern that in Brazil post-testing frequency of occurrence of diseases vectored by Aedes aegypti mosquitoes did not decrease:

> "I know that there has been some conflicting news on this experiment so I had decided to go straight to a source of information, a Brazilian newspaper[78]. This was to conclude as to whether or not the experiment worked in Brazil where the altered mosquitoes had been released in 2016. This paper is from Sao Palo, Brazil on September 11, 2019 and they are saying that there is an INCREASE of all three illnesses by Aedes aegypti mosquitoes: dengue, chikungunya, and our most feared zika. They have reported the statistics: " The number of cases of dengue, zika and chikungunya, diseases transmitted by the mosquito Aedes aegypti, increased in Brazil this year. In all, the three diseases caused 650 deaths as of December 30, 2018 and August 24, 2019. The Southern region had the highest percentage increase in new cases of the three diseases. The largest percentage increase was registered by dengue cases, a jump of 599.5%. As of August 24, there were 1,439,471 cases diagnosed in Brazil - or 690.4 cases per 100,000 inhabitants - and 591 deaths. In So Paulo, the state with the sharpest increase, the number of cases is 38 times higher than the previous year (3,712%), jumping from 11,475 to 437,047 cases. In Paran, the jump was 3,563%. Already cases of chikungunya went from 76,742 last year to 110,627 in 2019, registering a rate of 53.1 cases per 100,000 people. In the case of chikungunya, the state with the highest percentage change was Alagoas, 1,011%, a jump from 138 cases to 1,534. Regarding cases of Zika, the growth registered in Brazil was 47.1%, with 9,813 and 2 deaths. The incidence rate is 4.7 cases per 100 thousand inhabitants. The state with the largest percentage increase was Rio Grande do Sul, 1,083%. . . . Please do not release the GMO mosquitoes in the states of Florida and Texas. We really don't want to have an increase of mosquitoes and their illnesses like Brazil has!" (T. Ritchie 0223 p. 1-2) [Emphasis in the original]

The Center for Food Safety (0344) and GeneWatch UK (0335) stating that there "are no published peer-reviewed paper for Oxitec's GE Aedes aegypti OX5034 mosquitoes" indicated that necessary tests included:

> "A protocol for testing the GE mosquitoes for pathogenic agents prior to release." (GeneWatch UK 0335 p. 15-16)

4. Pathogenic Viruses Could Evolve in Response to OX5034

Friends of the Earth (0342) added that:

[78] https://gauchazh.clicrbs.com.br/geral/noticia/2019/09/casos-de-dengue-crescem-599-em-oito-meses-e-ministerio-da-saude-lanca-nova-campanha-ck0fndv9001fu01temrxb192g.html

> "Lastly, there is concern around the possibility of the dengue virus to evolve and become more potent and virulent in response to the introduction of the GE mosquitoes, and this could put human health at greater risk".[79] (Friends of the Earth 0342 p. 5)

B. Comments on Toxicity/Allergenicity of Proteins Engineered into OX5034

Some commenters expressed concern that OX5034 Aedes aegypti mosquitoes could pose risk to human health through toxicity or allergenicity given the potential for exposure through two possible routes, ingestion and injected mosquito saliva.

1. Potential Routes of Exposure

Some commenters expressed concern that OX5034 Aedes aegypti mosquitoes could pose risk to human health and identified two potential routes of exposure: through ingestion of Aedes aegypti mosquitoes carrying the tTAV-OX5034 and/or DsRed2-OX5034 proteins, or through bites from female Aedes aegypti mosquitoes carrying OX5034 genes encoding these proteins. Some commenters also requested more testing be done.

i. Potential for human exposure through the oral route

Some commenters (Friends of the Earth 0342, GeneWatch UK 0335) suggested that humans could be exposed to A. aegypti mosquitoes expressing the tTAV and/or DsRed2 proteins through the oral route, i.e., through ingestion of either larvae of flying adults.

Friends of the Earth (0342) stated that:

> "Given the high number of mosquitoes that are proposed for release, and based on experience in the Brazil, there is a high likelihood that humans or animals could swallow the GE mosquitoes upon release. As reported in Brazil, because of the high number of GE mosquitoes released, "... it's impossible to talk during the liberation sessions without accidentally swallowing a few..."[80] (Friends of the Earth 0342 p. 4-5)

Similarly, GeneWatch UK (0335) stated that:

> "Humans, animals and wildlife will also swallow adult GE mosquitoes. Journalists have reported that in Brazil, during experiments with Oxitec's OX513A GE mosquitoes, "*...it's*

[79] Medlock, J., Luz, Paula M., Struchiner, Claudio J., and Galvani, Alison P. (2009) The Impact of Transgenic Mosquitoes on Dengue Virulence to Humans and Mosquitoes. The American Naturalist 174, 565-577.

[80] Dengue, where is thy sting? LA Times. 1st November 2012. http://articles.latimes.com/2012/nov/01/world/la-fg-brazil-mutant-mosquitoes-20121102

> *impossible to talk during the liberation sessions without accidentally swallowing a few…"* due to the very large numbers of GE mosquitoes being released to try to swamp the wild population".[NOTEREF _Ref27058296 \h * MERGEFORMAT] (GeneWatch UK 0335 p. 13-14) [Emphasis in the original]

GeneWatch UK (0335) also pointed out that:

> "Because the female GE mosquitoes mostly die at the larval stage, there will be large numbers of dead GE larvae in the water where the female mosquitoes lay their eggs, and these might be ingested by humans, animals or wildlife." (GeneWatch UK 0335 p. 13-14)

ii. Potential exposure to humans though the saliva of the biting female mosquito

Two commenters (GeneWatch UK 0335, Friends of the Earth 0342) suggested that humans could be exposed to OX5034 proteins through the bite of a female Aedes aegypti mosquito.

GeneWatch UK (0335) commented that:

> " . . , people and animals may be bitten by female GE mosquitoes, if any survive or are inadvertently released." (GeneWatch UK 0335 p. 14)

Friends of the Earth (0342) stated that:

> "Also of concern is that biting female GE mosquitoes may inject a novel engineered protein into humans; Oxitec has yet to conduct or publish any study showing that this novel protein is not expressed in the mosquito's salivary gland." (Friends of the Earth 0342 p. 4-5)

2. Toxicity

Anonymous 0329 stated that:

> "GM mosquito expresses protein that has been shown to be toxic to more than just the Aedes aegypti. The tTA protein has been shown to be a toxin and neurotoxin to rodents, which are mammals, and therefore may be toxic to other mammals such as bats or even humans that might consume them. Signs of toxicity and neurotoxicity have been reported in mice expressing the tTA protein. Other mice studies have detected adverse effects on the lung." (Anonymous 0329 p. 1 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

GeneWatch UK (0335) provided references for these observations:

> "In the scientific literature, there is some evidence that enhanced tTAV expression can have adverse effects (loss of neurons affecting cognitive behaviour) in transgenic (GE) mice[81]. Other mice studies have detected adverse effects on the lung[82],[83]. These studies should act as warning signs that further evidence is needed." (GeneWatch 0335 p. 14)

GeneWatch UK (0335) stated that more information on the proteins engineered into OX5034 is needed:

> "However, other than a bioinformatics report (desk study), Oxitec has to date provided limited evidence regarding the safety of the RIDL genetic mechanism and the high level expression of tTAV that kills the insects at the larval stage. The mechanism of action of this killing mechanism is not fully understood and very limited safety data is available. The tetracycline transactivator (tTAV) protein is created by fusing one protein, TetR (tetracycline repressor), found in *Escherichia coli* bacteria, with the activation domain of another protein, VP16, found in the Herpes Simplex Virus. Researchers commonly use this mechanism to switch on and off different genetic traits, for example in transgenic (GE) mice used in medical research, but it is not normally present in the human food chain.

Gene Watch UK (0335) and Center for Food Safety (0344) noted that:

> " . . , it is unclear whether or not tTAV-OX5034 is the identical to the protein in the OX513A strain, and no studies specific to the OX5034 strain have been provided." (GeneWatch 0335 p. 14; Center for Food Safety 0344 p. 16)

3. Calls for More Testing and Information

GeneWatch UK (0335) and Center for Food Safety (0344), argued that additional testing is necessary:

> "Considerably more data, based on specific feeding trials in relevant species, is therefore needed to establish that consumption of OX5034 GE mosquito adults or

---

[81] Han HJ, Allen CC, Buchovecky CM, et al. (2012) Strain background influences neurotoxicity and behavioral abnormalities in mice expressing the tetracycline transactivator. *J Neurosci.* **32**(31):10574–10586. doi:10.1523/JNEUROSCI.0893-12.2012.

[82] Sisson TH, Hansen JM, Shah M, Hanson KE, Du M, Ling T et al. (2006) Expression of the Reverse Tetracycline-Transactivator Gene Causes Emphysema-Like Changes in Mice. *American Journal of Respiratory Cell and Molecular Biology*, **34**(5), 552 –560.

[83] Whitsett JA, Perl A-KT (2006) Conditional Control of Gene Expression in the Respiratory Epithelium: A Cautionary Note. *American Journal of Respiratory Cell and Molecular Biology*. **34**(5):519–520. http://www.atsjournals.org/doi/pdf/10.1165/rcmb.F310

> larvae is not harmful to humans, farm animals, pets or wildlife." (GeneWatch 0335 p. 14; Center for Food Safety 0344 p. 17)

GeneWatch UK (0335) and Center for Food Safety (0344) referred EPA to the standards developer by the European Union (EU) for GE insects indicating that these state:

> " . . . (page 8): "*...applicants should also assess the likelihood of oral exposure of humans to GM animals or their products which are not intended for food or feed uses. If such exposure is likely and ingestion or intake will occur at levels which could potentially place humans at risk, then applicants should apply the assessment procedures described in the EFSA Guidance Document on the risk assessment of food and feed from GM animals and on animal health and welfare aspects*". To meet the requirements of the cited Guidance on risk assessment of food and feed, it is likely that repeated dose toxicity studies using laboratory animals would be required."[84] (GeneWatch UK 0335 p. 14 Center for Food Safety 0344 p.17) [Emphasis in the original]

4. Allergenicity

Two types of comments were received on allergenicity potential: comments that expressed concern and those that express reassurance about safety.

i. Comments expressing concern

Anonymous (0329) stated that:

> "The proteins expressed by GM mosquitoes contain amino acid sequences identical to known human allergens." (Anonymous 0329 p.1 referencing: https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313)

Florida Keys Environmental Coalition (0331) stated that:

> "If females with modification are detected in measure statistical study, then allergen studies should be performed for these insects and clinical trial of the bite on humans must be performed. Oxitec has been negligent in performing any studies that evaluate the effect of the OX 513A bite and the same lack of knowledge should be avoided for the OX5034." (Florida Keys Environmental Coalition 0331 p.3)

GeneWatch UK (0335) indicated that some information that might be of relevance to the DsRed2 protein is available, albeit limited:

---

[84] EFSA (2012) Guidance on the risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects. EFSA Journal **10**(1):2501 [43pp]. http://www.efsa.europa.eu/en/efsajournal/pub/2501

> "In its application to release GE moths in New York State (since withdrawn but later resubmitted, although a brief open-release trial has now ceased), Oxitec provides a commercial reference for toxicity testing of the red fluorescent marker, DsRed2, by Pioneer DuPont.[85] Oxitec also cites a 26- day feeding study in rats, using GE oil seed rape (canola) genetically modified to express green (not red) fluorescent protein (GFP), which concludes: "*These data indicate that GFP is a low allergenicity risk and provide preliminary indications that GFP is not likely to represent a health risk*".[86] (GeneWatch UK 0335 p. 14) [Emphasis in the original]

ii. Comments expressing reassurance

R. E. Goodman (0337) stated that he had:

> " . . . evaluated potential allergenicity and toxicity of the first generation, OX513A, using bioinformatics as one would do for a GM plant, microbe or animal according to CODEX Alimentarius guidelines. There was no significant sequence to allergens or toxins, and that is the primary potential risk factor, identity matches to a known allergen or toxin. I have not worked on the second generation, but as it is described, I assume the proteins are still the same." (R. E. Goodman 0337 p.1)

C. Comments on Potential for Environmental Releases of OX5034 to Contribute to Increases in Antibiotic Resistance in Microbial Populations

Several commenters expressed concern that the use of antibiotics to produce products such as OX5034 could contribute to increases in antibiotic resistance in microbial populations. Some commenters described why an increase in resistance to antibiotics in microbial pathogen populations is a public health concern. Other comments offered hypotheses on how use of tetracycline to produce OX5034 mosquitoes could be a pathway to increases in antibiotic resistance in microbial populations. Other commenters offered explanations of why it is unlikely that use of tetracycline to produce OX5034 would affect antibiotic resistance in microbial pathogen populations.

Anonymous (0329) pointed out that:

> "Oxitec uses tetracycline in the breeding process . . . ." Anonymous 0329 p. 1-2 referencing: [HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313"])

Center for Food Safety (0344) and GeneWatch UK (0335) stated that:

---

[85] Pioneer Hi-Bred International (2006) Early Food Safety Evaluation for a Red Fluorescent Protein: DsRed2. 11th October 2006. http://www.fda.gov/downloads/Food/Biotechnology/Submissions/UCM219002.pdf

[86] Richards HA, Han CT, Hopkins RG, Failla ML, Ward WW, Stewart Jr. CN (2003). Safety assessment of recombinant green fluorescent protein orally administered to weaned rats. *Journal of Nutrition*, **133**(6), 1909–1912.

> "Oxitec feeds its GE mosquitoes on the antibiotic tetracycline, as this acts as a chemical switch to turn off the genetic killing mechanism." (Center for Food Safety 0344 p. 10; GeneWatch UK 0335 p. 8)

Friends of the Earth (0342) stated that:

> Oxitec's mosquitoes are engineered to be dependent on the presence of tetracycline and to die in its absence. In theory, the males will mate and the females die off while their tetracycline-dependent gene passes onto their offspring. The female offspring should die in the late larvae or pupae stage, . . ." (Friends of the Earth 0342 p. 3)

Center for Food Safety (0344) and GeneWatch UK (0335) stated that:

> "The use of tetracycline to breed the GE mosquitoes in the lab carries the risk of spreading antibiotic resistance, which could pose a major risk to human and animal health. (Center for Food Safety 0344 p. 10; GeneWatch UK 0335 p. 8)

J.W. Norris (0334) stated that:

> "When these mosquitoes are mass produced, it requires a lot of tetracycline to rear them as well as a lot of human employees to manufacture and distribute them. The fundamental design flaw of the RIDL system is that an antibiotic, when used by itself, will select for resistance." (J.W. Norris 0334 p. 1 of the Attachment)

1. Why Increases in Antibiotic Resistance in Microbial Pathogens is a Concern

J. W. Norris (0334) argued that increasing resistance to antibiotics in microbial pathogen populations is a public health problem that must be taken into account in assessing products of antibiotic dependent technologies such as that used to create OX5024. In a petition submitted to the docket, while recognizing the serious threat posed by mosquito borne diseases, he stated that:

> "The primary hesitation after review of the technology stemmed from the use of tetracycline as a necessary cofactor for mosquito development. Antibiotic resistance is another epidemic those in public health must consider in evaluation of this technology. In 2013, the CDC estimated that in the United States alone more than two million people are sickened every year with antibiotic-resistant infections, with at least 23,000 dying as a result.[87] These estimates are based on conservative assumptions and are likely minimum estimates.[NOTEREF _Ref29307578 \h * MERGEFORMAT] If the issue of antimicrobial resistance is not addressed by 2050, 10 million people are expected to die annually from

[87] Centers for Disease Control and Prevention, Office of Infectious Disease Antibiotic resistance threats in the United States, 2013. Apr, 2013. Available at: http://www.cdc.gov/drugresistance/threat-report-2013.

> resistant organisms.[88] The economic analysis of such a rise in resistance by 2050 has also lead to a predicted reduction of 2% to 3.5% in GDP and cost the world up to 100 trillion USD.[NOTEREF _Ref29309502 \h * MERGEFORMAT] ... For this reason, we need to ensure that our solution to these mosquito-borne illnesses does not exacerbate the global antimicrobial resistance epidemic." (Norris attachment 0334 p. 1)

Anonymous (0329) noted the importance of tetracycline in controlling certain microbial pathogens stating that:

> "Tetracycline is used to treat MRSA and its non-medical use may lead to tetracycline resistant MRSA." Anonymous 0329 p. 1-2 referencing: [HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313"])

2. Routes of Through Which Microbial Populations Might be Exposed to Tetracycline

Commenters raised the possibility that production and use of OX5034 might lead to increased antibiotic resistance in microbial populations, including in microbial pathogens, through two different pathways: through the disposal of tetracycline-containing waste materials from the OX5034 breeding process, or by the release of mosquitoes carrying resistant bacteria.

i. Potential for waste from breeding operations to lead to increased antibiotic resistance in microbial populations

Anonymous (0329) stated that:

> "The waste from this tetracycline has the potential to increase antibiotic resistant pathogens". Anonymous 0329 p. 1-2 referencing: [HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313"])

Center for Food Safety (0344) and GeneWatch UK (0335) stated that:

> "Disposal of waste water, containing tetracyclines and/or tetracycline-resistant bacteria, may also spread antibiotic resistance." (Center for Food Safety 0344 p. 10; GeneWatch UK 0335 p. 8)

---

[88] The Review on Antimicrobial Resistance, chaired by Jim O'Neill. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. Dec 2014. http://www.jpiamr.eu/wp-content/uploads/2014/12/AMR-Review-Paper-Tackling-a-crisis-for-the-health-and-wealth-of-nations_1-2.pdf

ii. Potential for releases of the tetracycline-exposed OX5034 mosquito to lead to increased antibiotic resistance in microbial populations

Several commenters questioned whether OX5034 mosquito's tetracycline-exposed microbiome could be a route to increased antibiotic resistance in microbial populations. One commenter described in detail how exposure to an antibiotic could affect a mosquito's microbiome beyond its use in production of OX5034 males.

Center for Food Safety (0344) and GeneWatch UK (0335) cited a reference indicating that resistant bacteria could be associated with OX5034:

> "A postgraduate student working with Oxitec's GE *Aedes aegypti* mosquitoes has conducted relevant experiments which found that "*Colonies grew on plates supplemented with 50 µg ml-1 of chlortetracycline, indicating that the larvae bore chlortetracycline-resistant bacteria*".[89] (Center for Food Safety 0344 p. 10; GeneWatch UK 0335 p. 8) [Emphasis in the original]

Center for Food Safety (0344 GeneWatch UK (0335) and Anonymous (0226) explained that "the use of tetracycline to breed the GM mosquitoes in the laboratory also carries the risk of spreading antibiotic resistance" because:

> "Insect guts are reservoirs for antibiotic resistance genes with potential for dissemination[90],[91]. Insect production in factories exposed to antibiotics could lead to drug resistance in their microbiota so that the insects disseminate antibiotic resistance when released into the environment[92],[93]." (Center for Food Safety 0344 p. 10; GeneWatch UK 0335 p. 8; Anonymous 0226 p. 11)

Center for Food Safety (0344) and GeneWatch UK (0335) stated that:

> "Oxitec's letter to the EPA states that released male OX5034 *Aedes aegypti* will be reared in the absence of tetracycline. This is not possible for the OX513A strain, but is possible for OX5034, because the latter strain is female-killing only (so male larvae do

[89] Ridley, E. V. (2011). The impact of chlortetracycline on *Drosophila melanogaster* and *Aedes aegypti* (PhD). University of York. Retrieved from http://etheses.whiterose.ac.uk/1642/1/Impact_of_chlortetracycline_on_Drosophila_melanogaster_and_Aedes_aegypti_EVRidley.pdf

[90] Zurek, L. and A. Ghosh (2014) Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. *Appl Environ Microbiol.* **80**(12): 3562-7.

[91] Allen, H.K., et al. (2009) Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants. *DNA Cell Biol.* **28**(3): p. 109-17.

[92] Tian, B., et al. (2012) Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. mBio, 3(6) :e00377-12.

[93] Levy, S.B. and B.M. Marshall (2013) Honeybees and tetracycline resistance. *mBio*, 4(1): e00045-13.

> not need to be fed the antibiotic in order to survive). However, the OX5034 strain requires tetracycline at the egg production stage as the female parents of the males intended for release need the antibiotic in order to survive to adulthood to lay their eggs. Thus, **there will likely be tetracycline-resistant bacteria in the egg stage of the GE males, which may persist until their release on adulthood.** (Center for Food Safety 0344 p. 10; most of this comment repeated by GeneWatch UK 0335 at p.8) [Emphasis in the original]

Center for Food Safety (0344) and GeneWatch UK (0335) added that:

> **There is also potential for intergenerational transfer of antibiotic resistant bacteria**, although we are not aware of any studies of this in *Aedes aegypti*." (Center for Food Safety 0344 p. 10; most of this comment repeated by GeneWatch UK 0335 at p.8) [Emphasis in the original]

Similarly, Anonymous (0329) stated that:

> GM mosquitoes exposed to tetracycline during the breeding process may pass antibiotic resistant bacteria to their offspring." Anonymous 0329 p. 1-2 referencing: [HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2017-0756-0313"])

J.W. Norris (0334) explained that:

> "The fact the OX5034 males do not physically contact tetracycline themselves in no way negates the previous concerns about promotion of antibiotic resistant bacteria and spread via OX5034 males to human environments of these resistant bacteria. The OX5034 breeding females require enough tetracycline to get into every cell of these genetically modified females to shut off the lethal proteins at the implanted genetic switch. Such exposure to an antibiotic will press the microbiome of the females to antibiotic resistant bacteria. When the OX5034 females lay eggs, they will share this pressed microbiome with their eggs. This is much the same way chicken eggs become contaminated with salmonella. Salmonella is screened for in chicken eggs, leading to chicken egg recalls. When the OX5034 eggs hatch they will contaminate the larva and the larval trays with these pressed bacteria. If allowed to mature and be released, these OX5034 males will search out wild female and mate with them. The act of mating will contaminate the wild females with the antibiotic pressed bacteria of the parent much like an sexually transmitted disease. When the wild female feeds on a human, there is concern about human contamination with resistant, possibly pathogenic bacteria." (J.W. Norris 0334 p. 1)

With regard to the possibility of pathogenic bacteria being transmitted, J.W. Norris (0334) stated that:

> "The water in which the mosquitoes are bred is not sterile, nor are the mosquito that are bred in them. Several images provided by news outlets from OX513A production facilities demonstrate hand contact without gloves, an employee placing his ungloved thumb in a larvae-filled container, . . , of greater significance is the seeding of larvae with bacteria from the employee's hand as well as the spread of bacteria from the tetracycline baths onto the employees hand. These tetracycline baths – from handling by human employees to the very fact living organisms are bred in them – have the potential to become major bacterial breeding sites." (Norris 0334 p. 1-2 of the Attachment)

J.W. Norris (0293) added that:

> "The fact that there is a mold issue in production suggests a hygiene issue greater than expected." (J.W. Norris 0293 p. 1)

J.W. Norris (0334) further explained the pathway through which he believes resistance to antibiotics could be increased through releases of OX5034 mosquitoes:

> "The process of pupal washing is not sufficient for the purpose of allaying resistance concerns. Once larva begin to pupate they are removed from the tetracycline baths and rinsed 4 to 6 times in fresh tap water. They are then placed in a tap water bath. This may be sufficient for removing excess tetracycline, but the bacterial film from the tetracycline bath must be expected to remain. The promotion of tetracycline resistant bacteria coating the external surface of each pupae must therefore raise concern the new bath will be contaminated by this bacteria." (J.W. Norris 0334 p. 2 of the Attachment)

J.W. Norris (0334) went on to explain that:

> "The emergence of the adult mosquito from its pupal case provides an additional concern for contamination of the adult. An adult Aedes aegypti first emerges from its pupal case by displaying its legs and pushing out of its external pupal casing. (Appendix B) Contamination would be unavoidable in the likely setting of resistant bacteria. Furthermore, Coon et al. showed using PCR that *E. coli* that had colonized axenic larvae was transstadially transmitted to adult *Ae. Aegypti*.[94] The fact molecular tetracycline is likely negligible is irrelevant as the future generation of bacteria do not have a lethal gene and can be expected to genetically have the inheritance of their tetracycline bath forbearers." (J.W. Norris 0334 p. 2 of the Attachment)

[94] Coon KL, Vogel KJ, Brown MR, Strand MR. Mosquitoes rely on their gut microbiota for development. Mol Ecol. 2014;23(11):2727-39.

Finally, J.W. Norris (0334) went on to explain that OX5034 mosquitoes may be able to transmit tetracycline resistant bacteria to human living environments:

> ". . . , there is also the possibility of spread of the bacteria by the mosquitoes themselves. Recent publication by Junqueira et al demonstrates flying insects can mechanically contaminate an environment with bacteria they receive in a previous environment.[95] This research was focused on the blow fly and the house fly but demonstrates capably the ability for promoted resistant bacteria from an environment such as the tetracycline maturation trays . . . to be transplanted mechanically to target areas such as people's homes . . . ." (J.W. Norris 0334 p. 2 of the Attachment)

3. Other Antibiotic Resistance Considerations

GeneWatch UK (0335) and the Center for Food Safety (0344) questioned whether antibiotics other than tetracycline (penicillin and streptomycin) were used to produce OX5024 and whether resistance to these antibiotics could also be driven by their use in OX5034 production. They stated that:

> "Protocols described in documents released in response to the GeneWatch UK Freedom of Information requests raise further questions about the use of antibiotics by Oxitec. The documents reveal that the company feeds its adult OX513A *Aedes aegypti* mosquitoes on sugar solution containing the antibiotics penicillin and streptomycin, during egg production (Section 1.2 of the Quality Control Protocol for the Assessment of Mating Competitiveness, page 88 of the pdf; and Section 1.2 of the Quality Control Protocol for Colony Genotyping, page 101 of the pdf).[96] . . . It is unclear from the information provided, whether penicillin and streptomycin are fed to adult GE mosquitoes only during specific experiments, or also during mass production, prior to open release into the environment." (GeneWatch UK 0335 at p. 8-9; Center for Food Safety 0344 p. 10)

4. Could Antibiotic Use Affect the Mosquito's Competency at Vectoring Pathogens

Two commenters expressed concern that antibiotics may affect Aedes aegypti competency to vector pathogens. These commenter, Center for Food Safety (0344) and GeneWatch UK (0335), stated that:

---

[95] Junqueira ACM, Ratan A, Acerbi E, Drautz-Moses DI, Premkrishnan BNV, Costea PI, et al. The microbiomes of blowflies and houseflies as bacterial transmission reservoirs. Sci Rep. 2017;7(1):16324.

[96] *Aedes aegypti* OX513A: Investigational Trial Protocol for the Evaluation of Efficacy. Oxitec July 2016 V12. http://www.genewatch.org/uploads/f03c6d66a9b354535738483c1c3d49e4/Investigational_field_protocol__PDF___REDACTED_.pdf

> "There is some evidence that antibiotics may increase the transmission of dengue fever by *Aedes aegypti* mosquitoes."[97] (GeneWatch UK 0335 p. 8-9; Center for Food Safety 0344 p. 10)

5. Calls for Additional Testing and Information

Center for Food Safety (0344) and GeneWatch UK (0335) stated that considerably more information is needed to confirm or rule out the presence of antibiotic resistant bacteria in the GE mosquitoes intended for release. For example, Center for Food Safety (0344) stated that:

> "More information is needed to be able to confirm or rule out the presence of such antibiotic resistant bacteria in the GE mosquitoes intended for release. Antibiotic resistant bacteria could pose a major risk to health if spread into the environment." (Center for Food Safety 0344 p. 10; most of this comment repeated by GeneWatch UK 0335 at p.8)

GeneWatch UK (0335) and the Center for Food Safety (0344) stated that:

> "Protocols described in documents released in response to the GeneWatch UK Freedom of Information requests raise further questions about the use of antibiotics by Oxitec.. . . It is unclear from the information provided, whether penicillin and streptomycin are fed to adult GE mosquitoes only during specific experiments, or also during mass production, prior to open release into the environment. This would raise additional concerns because:
> • The scale of the disposal problem would increase if these antibiotics are used during mass production;
> • It could lead to the spread of antibiotic resistant bacteria by the GE mosquitoes on release; . . . " (GeneWatch UK 0335 at p. 8-9; Center for Food Safety 0344 p. 10)

J.W. Norris (0334) stated that:

> "Our petition continues to request culture testing to assess this human health concern." (J.W. Norris 0334)

The Florida Keys Environmental Coalition (0331) stated that:

- "Mosquitoes, eggs and larva need to be submitted for independent antibiotic resistant bacteria evaluation by more than one objective agency, or qualified entity." (Florida Keys Environmental Coalition 0331 p. 3)

[97] Xi, Z., Ramirez, J. L., & Dimopoulos, G. (2008). The *Aedes aegypti* Toll Pathway Controls Dengue Virus Infection. *PLOS Pathogens*, **4**(7), e1000098. http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1000098

The Center for Food Safety (0344) and GeneWatch UK (0335) stating that there "are no published peer-reviewed paper for Oxitec's GE Aedes aegypti OX5034 mosquitoes" indicated that necessary tests included:

> "Laboratory studies of the potential for antibiotic resistant bacteria to be spread into the environment via adult mosquito releases or disposal of larval rearing water or other wastes from the mosquito production facility.
> (GeneWatch UK 0335 p. 15-16)

6. Comments Rebutting Concerns About the Potential for Releases of OX5034 to Increase Antibiotic Resistance in Microbial Populations

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) stated that the "potential for Oxitec's mosquito technology and its subsequent deployment to lead to increased risk of antibiotic resistance, is negligible" because:

> "Oxitec makes use of a small level of tetracycline-family antibiotics in the rearing of our 2nd generation mosquito eggs in its facility in the UK. Oxitec technology does not increase risk of antibiotic resistant bacteria in the environment where egg manufacture or releases are carried out (as confirmed by the FDA in 2016[NOTEREF _Ref27670801 \h * MERGEFORMAT]), and Oxitec will not be using any tetracycline or any other antibiotic in the US." (N. Rose 0341 p. 8)

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) furthering the argument that the "potential for Oxitec's mosquito technology and its subsequent deployment to lead to increased risk of antibiotic resistance, is negligible," added that:

> **"• No tetracycline or other antibiotics will be used in rearing of Oxitec's non-biting male mosquitoes for the pilot project in the Florida Keys; no tetracycline will be released into the environment in the US; Oxitec will have no tetracycline in the US. . . .**
> **"• The Oxitec male OX5034 mosquitoes reared for release in Florida will never have been in contact with tetracycline, and therefore the risk of spreading tetracycline-resistant bacteria is negligible."** (N. Rose 0341 p. 8) [Emphasis in the original]

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) explained that:

> "• Oxitec will use only a small level of doxycycline, a common, widely-used member of the tetracycline family, only in the UK, to rear females which will not be released (but which produce the mosquito eggs to be used in Florida).
> "• The amount of doxycycline that would be used in the UK to produce the females that would supply all the eggs needed for the EUP is less than 5 grams."

(N. Rose 0341 p. 8)

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) arguing that "existing human and agricultural uses of tetracyclines are far more likely to result in the spread of antibiotic-resistant bacteria, than Oxitec's very limited use of doxycycline outside of the USA" stated that the amount of antibiotic Oxitec would use:

> ". . . is about the equivalent of two 10-day courses of antibiotics to treat a normal infection. More than 6.5 million courses of doxycycline were prescribed in the US in 2016[98].
> • In addition, the EPA[99], federal and Florida state governments have approved the deployment of hundreds of tons of antibiotics into Florida's environment annually for agricultural and food production purposes[100].
> • Agricultural use of antibiotics in Florida alone is 88 million times more than what Oxitec will use in the UK."
> (N. Rose 0341 p. 8)

N. Rose, Head of Regulatory Science at Oxitec Ltd., (0341) pointed out that:

> "When the FDA approved Oxitec's 1st generation technology in 2016[NOTEREF _Ref27670801 \h * MERGEFORMAT], it considered Oxitec's use of antibiotics and determined that there is no risk to humans, animals or the environment from use in Oxitec's rearing processes in the US. Now Oxitec's 2nd generation technology does not use any tetracycline/doxycycline in the US for rearing of male mosquitoes for release into the environment." (N. Rose 0341 p. 8)

[98] https://clincalc.com/DrugStats/Drugs/Doxycycline
[99] EPA, Final Registration Decision for the New Use of the Active Ingredient Oxytetracycline Hydrochloride on Citrus Crop Group 10-10 (Dec. 7, 2018), www.regulations.gov/document?D=EPA-HQ-OPP-2015-0820-0031
[100] https://www.nature.com/articles/d41586-019-00878-4